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HUMAN IMMUNE RESPONSE TO DENGUE INFECTIONS

FINAL REPORT

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JUNE 30, 1991

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-86-C-6208

University of Massachusetts Medical Center
Worcester, Massachusetts 01655

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 30, 1991	Final Report 1 Jul 86 - 30 Jun 91

4. TITLE AND SUBTITLE

Human Immune Response to Dengue Infections

5. FUNDING NUMBERS

DAMD17-86-C-6208

61102A
3M161102BS13 AA
DA312059

6. AUTHOR(S)

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Massachusetts Medical Center
Worcester, Massachusetts 01655

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The purpose of this contract was to analyze human immune responses to dengue virus infections. To understand the role of immune responses in recovery from dengue virus infection and in the pathogenesis of dengue hemorrhagic fever and dengue shock syndrome, we have analyzed (i) human $CD4^+$ $CD8^-$ T cell responses to dengue virus, (ii) human $CD8^+$ $CD4^-$ T cell responses to dengue viruses, (iii) activation of T lymphocytes in vivo in dengue virus infections, (iv) dengue virus infection of human cell lines, (v) enhancement of dengue virus infection by antibodies to dengue virus, (vi) antibody-dependent enhancement of dengue virus infection mediated by bispecific antibodies, (vii) production of interferon by dengue virus-infected cells, and (viii) induction of interferon alpha from human lymphocytes by autologous dengue virus-infected monocytes.

14. SUBJECT TERMS

Dengue virus; Cytotoxic T lymphocytes, T cell epitopes; Antibody-dependent enhancement

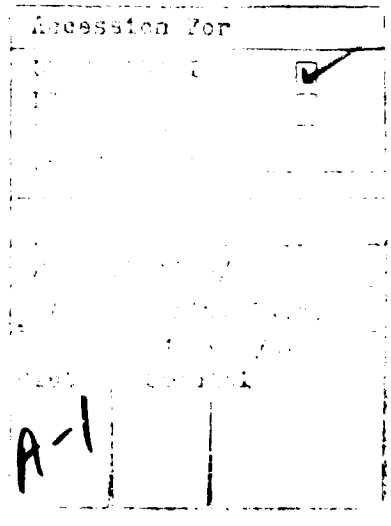
15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRAC
Unclassified	Unclassified	Unclassified	Unlimited

13. Abstract (continued)

Human $CD4^+$ and $CD8^+$ memory T lymphocytes generated by primary dengue virus infection were serotype-crossreactive at the clonal level. Dengue virus-specific $CD4^+$ T cell clones lysed dengue virus-infected autologous cells in an HLA class II-restricted fashion, and produce IFNγ. Dengue virus-specific $CD8^+$ T cell clones lysed dengue virus-infected autologous cells in an HLA class I-restricted fashion. Most of these $CD4^+$ and $CD8^+$ T cell clones recognized NS3 protein. IFNγ produced by $CD4^+$ T cells augmented infection of monocytic cell lines by dengue virus-antibody complexes. This augmentation was due to upregulation of $Fc_\gamma R$ expression by IFNγ. $CD4^+$ and $CD8^+$ T lymphocytes are activated <u>in vivo</u> during dengue virus infections, and the levels of T cell activation is greater in DHF than in DF. These results suggest that dengue virus-specific T lymphocytes contribute to recovery from dengue virus infection and also contribute to the immunopathology of DHF/DSS.



FOREWORD

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

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I. INTRODUCTION

Dengue viruses are the members of the family Flaviviridae, and there are four serotypes, dengue virus types 1, 2, 3 and 4. The virion consists of a single stranded RNA genome surrounded by a nucleocapsid, which is covered by a lipid envelope containing two proteins, envelope (E) and membrane (M) proteins. The RNA genome of dengue virus codes for three structural proteins, capsid (C), membrane (M), and envelope (E), and seven non-structural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (1).

Dengue viruses are transmitted to humans by mosquitos, mainly by Aedes Egypti mosquitos (2). The course of infection after a mosquito bite is not well understood; however, it is believed that monocytes/macrophages support dengue virus infection in vivo (3,4).

Dengue virus infections can be asymptomatic or cause two forms of illness, dengue fever (DF) and dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) (5). Dengue fever is a self-limited febrile disease which is characterized by fever, retro-orbital pain, muscle aches, bone pain and petechiae. Patients recover from infection in about a week without complications. In some situations, patients infected with dengue virus leak plasma into interstitial spaces resulting in hypovolemia and sometimes circulatory collapse. This severe and life-threatening syndrome, which is always accompanied by thrombocytopenia and sometimes by frank hemorrhage is termed dengue hemorrhagic fever (DHF). The World Health Organization categorized DHF cases into 4 grades, from less serious (grade 1) to severe (grade 4). Grades 3 and 4, in which plasma leakage is so profound that shock occurs, are also referred to as dengue shock syndrome (DSS) (6). The pathogenesis of DHF/DSS is not understood.

Dengue virus infections are a serious cause of morbidity and mortality in many areas of the world; Southeast and South Asia, Central and South America, the Caribbean and Africa. Dengue virus infections can be estimated to occur in up to 100 million individuals yearly. Since DHF/DSS was recognized in the 1950s, 1.5 million cases have been hospitalized, and 33,000 infected individuals have been reported to have died because of this syndrome (4). Therefore, dengue virus infections are one of the most important human infectious diseases.

Immune responses to dengue viruses have been studied in humans, monkeys and mice infected with dengue viruses. It is thought that immune responses may have two opposite roles in dengue virus infections, (i) prevention of dengue virus infection and recovery from infection and (ii) the immunopathology of DHF/DSS.

The role of immune responses in the prevention of infection

and in recovery from dengue virus infection has been suggested by virus challenges of humans and mice. It has been reported that humans infected with one serotype of dengue virus maintain life-long protective immunity to infection by the homologous serotype, although protective immunity to infection by heterologous serotypes is short-lived (7). Immunization of mice with one serotype of dengue virus protects mice from lethal challenge infection with the same serotype of dengue virus (8). Although the mechanisms of these protective immune responses have not been clearly understood, these observations indicate that immune responses to dengue viruses contribute to the prevention of secondary infections with dengue virus of the homologous serotype and probably contribute to recovery from infection.

The role of immune responses in the pathogenesis of DHF/DSS has been mainly suggested by epidemiological observations. In Thailand, patients who develop DHF/DSS can be divided into two groups: children over 1 year of age who have secondary immune responses, and children less than 1 year of age who have primary antibody responses (4,5). Patients who are over 1 year of age comprise approximately 90% of all the DHF/DSS cases (4). These cases are observed in secondary infections by a dengue virus of a different serotype from the dengue virus which caused the primary infections. On the other hand, children who are less than 1 year of age and develop DHF/DSS during primary infections are infants born to dengue antibody-positive mothers (4,5). These observations have suggested that the presence of dengue virus antibody and memory T cells may play important roles in the pathogenesis of DHF/DSS. Therefore, immune responses in dengue virus infection seem to be protective or immunopathological.

To understand the role of immune responses in dengue virus infections, we have analyzed human immune responses to dengue viruses, especially focusing on dengue virus-specific human T cell responses. We have found that dengue virus-specific $CD4^+$ $CD8^-$ T lymphocytes and $CD8^+$ $CD4^-$ T lymphocytes are generated after dengue virus infections. These T lymphocytes are activated <u>in vivo</u> during dengue virus infections, and activation is greater in DHF/DSS than in DF.

In addition to analysis of human T cell responses to dengue viruses, we have studied dengue virus infection of human mononuclear cells and fibroblasts. These cells can be infected with dengue virus and produce IFNα or IFNβ, which protect other uninfected cells from dengue virus infections. Furthermore, we have studied antibody-dependent enhancement of dengue virus infection. FcγRI and FcγRII mediate antibody-dependent enhancement of dengue virus infection, and IFNγ augments FcγRI-mediated dengue virus infection by upregulation of FcγRI. We have analyzed various aspects of interactions between human immune systems and dengue viruses for these 5 years. I will describe our observations in the results section.

II. RESULTS

A. Human CD4+ T cell responses to dengue virus infection

A-1. Proliferative responses of PBMC from dengue-immune donors induced by dengue antigens

PBMC from a dengue antibody-positive donor were cultured with dengue or control antigens diluted at various concentrations, and ^{3}H-TdR incorporation was examined. Dengue antigens induced significant proliferative responses of PBMC, and there was a good correlation between the level of the proliferative responses and the concentration of dengue antigens (Figure 1). Control antigen did not induce significant proliferative responses.

The results shown in Figure 2 illustrate the proliferative responses of PBMC obtained from eleven Thai donors and the two American donors who were known to have previously been infected with dengue virus. The PBMC from these donors showed significant proliferative responses induced by dengue antigens (8/9 with dengue 1 Ag; 10/13 with dengue 2 Ag; 5/7 with dengue 3 Ag; 5/7 with dengue 4 Ag). The PBMC from Massachusetts blood bank donors did not significantly proliferate in response to dengue antigens.

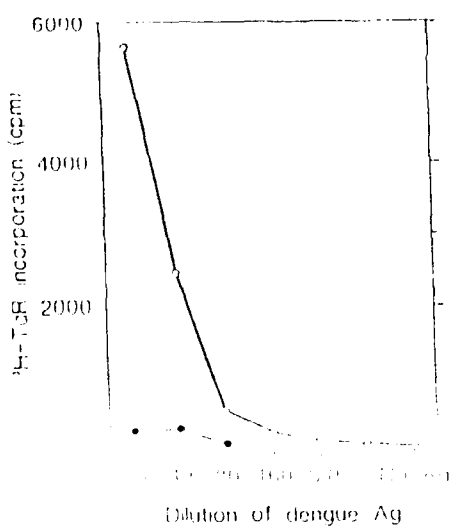


Figure 1: Proliferative responses of dengue immune PBMC. ○: proliferative responses induced by dengue-3 Ag. ●: proliferative responses induced by control Ag.

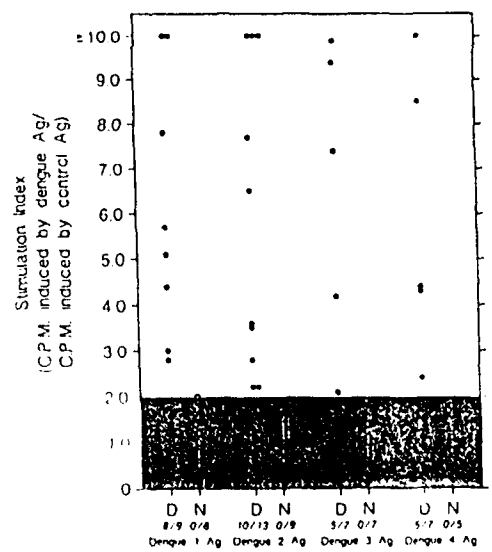


Figure 2: Proliferative responses of PBMC from 11 Thai and 2 American antibody-positive donors. Results were expressed as a stimulation index. D, dengue immune donors. N, nonimmune donors.

Table 1 contains a summary of the positive proliferative responses induced by dengue antigens using PBMC from Thai and American donors. These results indicate that antigens of the four dengue serotypes can induce proliferative responses using PBMC from dengue antibody-positive donors. Neither dengue antigens nor control antigens induced significant proliferative responses of PBMC from non-immune donors.

Table 1: A summary of proliferative responses of PBMC from dengue immune donors to dengue antigens*

	Dengue-immune** ^{3}H-TdR incorporation			Non-immune ^{3}H-TdR incorporation		
	No. of donors	Stimulation Index	c.p.m.	No. of donors	Stimulation Index	c.p.m.
Dengue 1	8	7.4 (2.8-15.7)	9011 (1018-19343)	6	1.0 (0.5-2.0)	664 (142-2357)
Dengue 2	10	7.5 (2.2-19.5)	4644 (1019-10872)	9	1.1 (0.6-1.4)	1038 (93-2897)
Dengue 3	5	6.6 (2.1-9.9)	6005 (1706-13762)	7	1.1 (0.7-1.9)	732 (158-1975)
Dengue 4	5	8.2 (2.4-21.5)	7936 (2153-17178)	5	1.3 (0.8-1.8)	611 (185-1275)
Control Ag	11	-	1046 (68-4033)	9	-	783 (261-2172)
No Ag	11	-	630 (69-1080)	9	-	529 (95-1723)

*4 x 10^5 PBMC were cultured for 6 days with dengue or control Ag diluted at 1:30. Cells were pulsed with 1.25 uCi ^{3}H-TdR for 8 hours before harvest. Results are presented as averages.

**The responses of PBMC of dengue-immune donors who had a stimulation index of greater than two with each of the four dengue antigens are included. The stimulation index was calculated from mean CPM induced by dengue Ag/mean CPM induced by control Ag. Quadruplicate samples were used.

A-2. Detection of IFNγ in the culture fluids of PBMC stimulated with dengue antigens

We examined the culture fluids of PBMC stimulated with dengue antigens for IFNγ using ELISA with monoclonal antibodies to human IFNγ and α. IFNγ was detected at high titer in the culture fluids of PBMC from dengue-immune donors stimulated with dengue antigens ($p<0.02$ compared to the amount of IFN detected in cultures of PBMC from non-immune donors), but not in the culture fluids of PBMc stimulated with control antigen or cultured alone (Table 2).

Table 2: IFNγ production by PBMC from dengue antibody-positive donors after stimulation with dengue antigens*

Donors	IFN (U/ml**					
	Dengue 1 Ag	Dengue 2 Ag	Dengue 3 Ag	Dengue 4 Ag	Control Ag	No Ag
Dengue antibody-positive						
T1	25	120	28	70	2	2
T2	42	56	51	115	14	14
T3	33	47	14	26	1	3
A1	32	43	61	38	3	3
Dengue antibody-negative						
M1	8	5	8	5	5	6
M2	3	11	4	3	2	3
M3	<1	2	2	2	2	2
M4	<1	<1	<1	<1	<1	<1

*4×10^5 PBMC were cultured for 6 days with dengue and control Ag diluted at 1:30. Culture fluids were examined for IFN and IFN by ELISA.

**The titers of IFN induced by dengue and control antigens were compared by Student's t test between dengue antibody-positive and antibody-negative donors. IFN induced by dengue 1 Ag, $p<0.001$; by dengue 2 Ag, $p<0.02$; by dengue 3 Ag, $p<0.02$; by dengue 4 Ag, $p<0.01$; by control Ag, $p>0.02$ (not significant); without Ag, $p>0.02$ (not significant).

A-3. Characterization of the lymphocytes responding to dengue antigens

We cultured the proliferating lymphocytes with γ-irradiated autologous PBMC in the presence of dengue antigens and IL-2 for 7 or 14 days, after the original 7 days of bulk culture stimulation, in order to characterize the lymphocytes which responded to dengue antigen. Phenotypic analyses showed that the proliferating cells

were predominantly $CD3^+$, $CD4^+$, $CD8^-$, $CD16^-$ and $CD20^-$ (Table 3). Therefore, the proliferating cells are T cells with the helper/inducer phenotype. They produced IFNγ after stimulation with dengue antigens, but not after stimulation with control antigen (Table 4).

Table 3: Phenotypes of short term-cultured T cell lines stimulated with dengue antigens

Donor	Ag	days of culture	% positive cells CD3	CD4	CD8	CD16	CD20
A1	dengue 3	14	89	84	2	2	<1
		21	91	83	3	ND	ND
T3	dengue 2	14	74	46	24	ND	ND
T4	dengue 2	14	84	80	8	ND	ND

Table 4: Proliferative responses and IFNγ production by a short-term cultured T cell line after stimulation with dengue antigens*

Culture days	^{3}H-TdR incorporation (cpm) Dengue Ag	Control Ag	No Ag	IFN (U/ml) Dengue Ag IFNγ	IFNα	Control Ag IFNγ	IFNα	No Ag IFNγ	IFNα
14	1325	250	270	40	<10	<1	<10	<1	<10
21	1290	380	432	280	<10	12	<10	10	<10

*5 x 10^4 short-term cultured T cells were cultured with 2.5 x 10^5 -irradiated autologous PBMC in 0.2 ml RPMI containing dengue 3 Ag or control Ag diluted at 1:30 for 3 days. Cells were pulsed with 1.25 uCi ^{3}H-TdR for 8 hours before harvest. Culture fluids were examined for IFN and IFN by ELISA.

A-4. Establishment of $CD4^+$ T cell clones which respond to dengue 3 antigen

The PBMC from donor A who had been infected 1 year earlier with dengue virus type 3 were cultured with dengue antigens of four serotypes, and ^{3}H-TdR incorporation was examined. PBMC from this donor responded to dengue 3 antigen, and they also responded to dengue 1, 2 and 4 antigens to lower but significant levels. PBMC from donor B who had been infected with dengue 1 virus about 4 months earlier responded best to dengue 1 antigen and also responded to dengue antigens of other serotypes to lower levels (Table 5).

Table 5: Proliferation responses of the PBMC of donor A to dengue antigens in bulk cultures*

Antigens	^{3}H-TdR incorporation (c.p.m.) Donor A**	Donor B	Donor C
Dengue 1	5128	25932	481
Dengue 2	6643	5983	516
Dengue 3	25177	6772	682
Dengue 4	2883	4329	603
Control	660	1065	460
No Ag	707	1091	450

*2 x 10^5 PBMC were cultured with dengue and control antigens diluted at 1:30 for 6 days. Cells were pulsed with 1.25 uCi 3H-TdR for 8 hours and ^{3}H-TdR incorporation was counted.

**Donor A was known to have been infected with dengue 3 virus. Donor B was known to have been infected with dengue 1 virus. Donor C is from Massachusetts and does not possess any anti-dengue antibodies.

A-5. Serotype specificity of the dengue-specific T cell clones

We tried to establish dengue-specific T cell clones by a limiting dilution method using lymphocytes from donor A and dengue 3 antigen. Twelve clones were established which respond to dengue 3 antigen, but not to control antigen. All the clones were established from wells containing 1 cell/well. The cloning efficiency was 15% with 1 cell/well, 48% with 3 cells/well, 90% with 10 cells/well and 100% with 30 cells/well. The clonality of the clones used in the experiments was greater than 96%. Phenotypic analysis of the clones using monoclonal antibodies showed that all the clones have $CD3^+$, $CD4^+$ and $CD8^-$ phenotypes (Table 6).

These clones were examined for serotype specificity using dengue antigens of four serotypes and yellow fever antigen. A dose response study using clone JK31 indicated that each of the antigens induced maximum proliferative responses at a 1:30 dilution (data not presented). Eight clones responded to dengue 1, 2 and 4 antigens to about the same level as to dengue 3 antigen (Table 7), and therefore they are dengue serotype cross-reactive. Three of these serotype cross-reactive clones (JK27, JK32 and JK35) also responded to yellow fever virus antigens. Four other clones responded predominantly to dengue 3 antigens, although there are some minor responses to other serotypes; therefore, they are serotype-specific.

Table 6: Phenotypic analysis of dengue virus antigen-specific T cell clones*

Clones	% antigen-positive cells		
	CD3	CD4	CD8
JK24	97	96	1
JK26	94	96	2
JK27	97	95	0.4
JK28	93	96	0.4
JK30	98	85	0.8
JK31	99	99	0.2
JK32	99	96	3
JK33	97	95	0.2
JK34	98	92	4
JK35	99	98	4
JK36	80	94	2
JK37	98	96	1

*Clones were stained with anti-CD3 (anti-Leu4), anti-CD4 (anti-Leu3) and anti-CD8 (anti-Leu2) monoclonal antibodies. The percentage of positive cells was counted on a FACS.

Table 7: Proliferative responses of dengue virus antigen-specific T cell clones to dengue and yellow fever (YF) antigens*

Clones	3H-TdR incorporation (c.p.m.)						
	Dengue 1	Dengue 2	Dengue 3	Dengue 4	YF	Control Ag	No Ag
Serotype cross-reactive							
JK26	1438	2078	2375	1394	642	436	708
JK27	6683	5711	11728	3839	1880	827	650
JK28	1949	2286	2600	1681	788	684	633
JK32	1416	3290	4102	2410	1925	133	245
JK33	4530	8152	12756	6286	3070	1161	1949
JK34	2651	8457	9256	4253	1910	480	1076
JK35	3507	4372	6260	5025	3854	2403	1290
JK36	1732	13159	15055	7167	903	140	1225
Serotype specific							
JK24	2581	2205	13809	1457	355	348	108
JK30	821	896	3246	721	276	112	218
JK31	745	1269	7309	837	397	140	133
JK37	4478	6958	26892	5827	2546	1223	1985

*1 x 10^4 cells were cultured with 2 x 10^5 γ-irradiated autologous PBMC in 0.2 ml RPMI/10% human AB serum containing dengue, yellow fever and control antigens diluted at 1:30 for 72 hours. Cells were pulsed with 1.25 uCi ^{3}H-TdR for 8 hours before harvest.

A-6. Production of IFNγ by dengue-specific T cell clones after stimulation with dengue antigens

The dengue-specific clones were examined for IFN production after stimulation with dengue and yellow fever antigens (Table 8). All the serotype cross-reactive clones produced IFN after stimulation with dengue 3 antigen, and all but JK27 produced IFNγ to the same or lower levels after stimulation with dengue antigens of the other serotypes. Clone JK35, which responded to yellow fever antigen, produced IFNγ after stimulation with yellow fever antigen. Serotype-specific clones produced IFNγ after stimulation with dengue 3 antigen (data not presented). These results suggest that IFNγ is produced by serotype cross-reactive T cells during secondary infections with different serotypes of dengue virus from primary infections.

Table 8: IFNγ production by serotype cross-reactive T cell clones after stimulation with dengue antigens*

	IFNγ (U/ml)						
Clones	Dengue 1	Dengue 2	Dengue 3	Dengue 4	YF	Control Ag	No Ag
JK26	26	74	39	19	9	6	7
JK27	7	6	35	4	3	3	3
JK28	13	14	13	6	2	1	<1
JK32	12	23	18	11	6	3	3
JK33	14	24	41	17	8	2	5
JK34	6	22	23	10	4	2	3
JK35	17	33	51	21	10	<1	3
JK36	2	15	18	7	2	3	<1

*1 x 10^4 cells were cultured with 2 x 10^5 γ-irradiated autologous PBMC in 0.2 ml RPMI/10% human AB serum containing dengue, yellow fever and control antigens diluted at 1:30 for 72 hours. Culture fluids were collected and assayed for IFN by ELISA.

A-7. Lysis of dengue 2 virus-infected autologous lymphoblastoid cell line by dengue serotype cross-reactive T cell clones

We examined other clones for their cytotoxic activities to dengue 2 virus-infected autologous LCL. All the cross-reactive clones but one (JK27) lysed dengue 2 virus-infected autologous LCL (Table 9). These clones did not lyse uninfected LCL or K562 cells. Dengue 3 serotype-specific clones did not lyse dengue 2-infected LCL, uninfected LCL or K562. These results suggest that serotype cross-reactive T cells may lyse dengue-infected cells during secondary infections.

Table 9: Lysis of dengue 2 virus-infected autologous LCL by serotype cross-reactive T cell clones*

Clones	Effector: target ratio	T Specific 51Cr release: Dengue-2 infected autologous LCL	Uninfected autologous LCL	K562
Serotype cross-reactive				
JK26	2	71	0	0
JK27	2	0	0	0
JK28	2	85	0	0
JK32	2	42	2	0
JK33	2	16	0	1
JK34	2	43	0	0
JK35	2	35	0	1
JK36	2	59	0	0
Serotype specific				
JK30	3	0	0	0
JK31	3	0	0	0
JK37	4	2	2	0

*2.5 x 10^3 target cells were incubated with effector cells for 4 hours. % specific ^{51}Cr release was calculated by the formula described in Materials and Methods.

A-8. Virus- and dengue serotype-specificity of $CD4^+$ T cell clones

These clones were examined for their virus- and dengue serotype-specificities in cytotoxic activities (Table 10). JK21 and JK37 lysed target cells cultured with dengue-3 Ag, but did not lyse target cells cultured with dengue-1, -2, -4, YFV or WNV Ag. Therefore, they are dengue serotype-specific. JK36 and JK46 lysed target cells cultured with dengue-2, -3 and -4 Ag, but did not lyse target cells cultured with dengue-1, YFV or WNV Ag. JK44 lysed target cells cultured with dengue-1, -2, and -3, but did not lyse target cells cultured with dengue-4, YFV or WNV Ag. Therefore, these three clones are dengue subcomplex-specific. Four clones, JK32, JK34, JK39 and JK41, lysed target cells cultured with dengue Ag of four serotypes, but did not lyse target cells cultured with YFV or WNV Ag. Therefore, they are dengue serotype-crossreactive.

JK28 lysed target cells cultured with dengue Ag of four serotypes and WNV Ag, but did not lyse target cells cultured with YFV Ag. JK26, JK43 and JK49 lysed target cells cultured with dengue Ag of four serotypes, YFV and WNV Ag. Therefore, these clones are flavivirus-crossreactive. These results indicate that dengue virus-specific $CD4^+$ T cells are heterogeneous in virus- and dengue serotype-specificity, and that there are at least six patterns of specificities.

Table 10: Virus- and dengue serotype-specificity of CD^+ $CD8^-$ T cell clones established from donor A infected with dengue-3 virus*

	% specific ^{51}Cr release							
Clones	Dengue 1	Dengue 2	Dengue 3	Dengue 4	Yellow Fever	West Nile	Control Ag	No Ag
Dengue serotype-specific								
JK21	1	0	20	0	0	0	0	0
JK37	1	0	57	0	0	0	0	0
Dengue subcomplex-specific								
JK36	0	55	50	15	0	0	0	0
JK46	1	68	67	27	0	0	0	0
JK44	24	65	34	0	0	0	0	0
Dengue serotype-crossreactive								
JK32	52	65	66	55	7	8	10	10
JK34	28	54	52	23	0	0	0	0
JK39	39	62	67	31	0	0	0	0
JK41	12	43	39	12	0	0	0	0
Flavivirus-crossreactive								
JK28	24	46	41	25	0	23	0	0
JK26	23	40	41	19	19	55	0	0
JK43	22	31	34	27	37	43	0	0
JK49	34	60	57	26	29	50	0	0

*2.5×10^3 target cells were incubated with effector cells for 6 hours. Percent specific ^{51}Cr release was calculated by the formula described in Materials and Methods. Effector/target ratio was 3:1 for JK21 and JK43, 4:1 for JK41, 6:1 for JK36, JK37, JK46, JK44, JK32, JK34, JK39 and JK49, 7:1 for JK26, and 12:1 for JK28. Underlines indicate significant levels of lysis.

A-9. HLA-restriction in the lysis of target cells by $CD4^+$ T cell clones

HLA restriction of the lysis of target cells by dengue virus-specific $CD4^+$ T cell clones were examined using monoclonal antibodies to HLA molecules (Table 11). Monoclonal Ab to HLA DP inhibited the lysis of target cells by a dengue-3-specific clone JK37, dengue serotype-crossreactive clones JK32, JK34, JK39 and JK41, and flavivirus-crossreactive clones JK26, JK28 and JK49. Monoclonal Ab to HLA DQ inhibited the lysis of target cells by dengue subcomplex-specific clones JK36 and JK46. Monoclonal Ab to HLA DR inhibited the lysis of target cells by a dengue-3-specific clone JK21 and a subcomplex-specific clone JK44. Interestingly, the lysis of target cells by a flavivirus-crossreactive clone JK43 was not inhibited by any of the 3 monoclonal Abs to HLA class II

nor an antibody to HLA class I. However, the lysis by JK43 was inhibited by a mixture of anti-HLA DP, HLA DQ and HLA DR Abs, and by an antibody to CD3 (data not presented).

These results indicate that dengue virus-specific $CD4^+$ T cell clones are HLA class II-restricted and that HLA DP, DQ and DR are used as restriction elements by the various clones.

Table 11: HLA restriction of lysis of dengue-3 Ag-cultured target cells by $CD4^+$ T cell clones*

	% specific ^{51}Cr release			
	None	Anti-HLA DP	Anti-HLA DQ	Anti-HLA DR
Dengue serotype-specific				
JK21	22	21	26	6
JK37	55	5	48	26
Dengue subcomplex-specific				
JK36	25	26	4	20
JK46	45	47	8	41
JK44	51	46	54	14
Dengue serotype-crossreactive				
JK32	83	7	80	71
JK34	87	1	83	77
JK39	46	1	48	45
JK41	56	7	47	40
Flavivirus-crossreactive				
JK28	91	11	78	77
JK26	64	1	64	69
JK43	56	49	55	59
JK49	53	1	41	57

*2.5×10^3 target cells were incubated with effector cells for 6 hours in the presence of monoclonal antibodies at final dilution of 1:80. B7/21.7, S3/4 and OKIa1 were used as anti-HLA DP, anti-HLA DQ and anti-HLA DR, respectively. Effector/target ratio was 4:1 for JK43, 5:1 for JK21, JK39 and JK46, 6:1 for JK37, JK26, JK32, JK44, and JK49, 7:1 for JK36, 11:1 for JK28, 14:1 for JK34, and 15:1 for JK41. Underlines indicate significant inhibition by each antibody.

A-10. Recognition of NS3 by dengue virus-specific T cell clones

We have previously reported that NS3 induces high levels of proliferation responses of donor A PBMC in bulk cultures (9). We next tried to determine whether $CD4^+$ T cell clones recognize purified NS3 protein (Table 12). Dengue-3-specific clone JK37, subcomplex-specific clones JK36 and JK46, and serotype-

crossreactive clones JK32, JK34 and JK39, lysed target cells cultured with NS3 protein purified from dengue-3 infected cells, but did not lyse target cells cultured with NS1 protein purified from dengue-3 virus-infected cells or NS3 from WNV-infected cells. A flavivirus-crossreactive clone JK43 lysed target cells cultured with NS3 obtained from dengue-3 infected cells or from WNV-infected cells, but did not lyse target cells cultured with NS1 obtained from dengue-3 virus-infected cells. Lysis of target cells cultured with the NS3 protein of dengue-3 by JK34 and JK39 was inhibited by antibody to HLA DP (data not presented). JK26, JK28, JK41, JK44 and JK49 did not lyse target cells cultured with NS1 or NS3 proteins (Table 12). These results suggest that the NS3 protein contains multiple epitopes recognized by dengue virus-specific $CD4^+$ T cells of various serotype-specificities. Table 13 gives a summary of these results.

Table 12: Recognition of NS3 protein by dengue virus-specific $CD4^+$ T cell clones*

	% specific ^{51}Cr release						
	Dengue-3** NS1	Dengue-3 NS3	West Nile NS3	Control cell protein	Dengue-3 Ag	West Nile Ag	No Ag
Dengue serotype-specific							
JK37	2	13	2	ND	56	0	1
Dengue subcomplex-specific							
JK36	10	40	ND	15	87	ND	19
JK46	7	20	0	ND	75	ND	7
JK44	0	1	0	ND	47	0	0
Dengue serotype-crossreactive							
JK32	11	27	10	14	77	10	6
JK34	2	14	0	0	79	0	0
JK39	7	19	0	ND	42	ND	6
JK41	0	3	ND	4	66	ND	0
Flavivirus-crossreactive							
JK28	2	7	0	1	70	30	0
JK26	0	5	0	0	60	49	0
JK43	7	25	84	8	78	93	0
JK49	7	5	0	2	77	72	0

*2.5×10^3 target cells incubated with effector cells for 6 hours. Effector/target ratio was 4:1 for JK32, 6:1 for JK26, JK37, JK39, and JK34, and 11:1 for JK36. Underlines indicate significant levels of lysis.

**Autologous EBV-transformed cells were cultured with dengue-2 NS1, dengue-3 NS3, West Nile NS3 and control cell protein at final concentration of 20 ug/ml, and dengue-3 Ag and West Nile virus Ag at 1:80 for 24 hours prior to addition of effector T cells.

Table 13: Summary of dengue virus-specific $CD4^+$ $CD8^-$ T cell clones

Virus- and serotype-specificity	Clones	HLA-restriction	Protein recognized* NS3	NS1
Serotype-specific				
D3	JK21	DR	not tested	
	JK37	DP	+	-
Subcomplex-specific				
D2, D3, D4	JK36	DQ	+	-
	JK46	DQ	+	-
D1, D2, D3	JK44	DR	-	-
Dengue serotype-crossreactive				
D1, D2, D3, D4	JK32	DP	+	-
	JK34	DP	+	-
	JK39	DP	+	-
	JK41	DP	-	-
Flavivirus-crossreactive				
D1, D2, D3, D4, WNV	JK28	DP	-	-
D1, D2, D3, D4, YFV, WNV	JK26	DP	-	-
	JK43	undetermined	+	-
	JK49	DP	-	-

*+ denotes recognition and - denotes no recognition.

A-11. Localization of epitopes on NS3 determined using dengue-vaccinia recombinant viruses

We attempted to localize epitopes recognized by these clones on NS3, using dengue-vaccinia recombinant viruses. Recombinant vaccinia virus #F which contains the entire NS3 genomes, #E which codes for amino acid (a.a.) residues 1-452 and NS3, #G which codes for a.a. residues 1-182 of NS3, and #X which codes for a.a. residues 453-618 were prepared by Dr. C.-J. Lai of NIAID, NIH. JK32 lysed autologous lymphoblastoid cell line (LCL) infected with #F and #E, but did not lyse those infected with #X. JK34 lysed LCL infected with #F and E#, but did not lyse those infected with #G or #X. JK43 lysed LCL infected with #F, #E and #G, but did not lyse LCL infected with #X (Table 14). These results indicate that the epitope recognized by JK32 is located within a.a. 1-452, the epitope recognized JK34 is located within a.a. 183-452 of NS3, and the epitope recognized by JK43 is located within a.a. 1-182 of NS3.

Table 14: Localization of epitopes recognized JK34 and JK43 on NS3

Target cells infected with re-vaccinia virus (VV)	% specific ^{51}Cr release*		
	JK32	JK34	JK43
#D = vv (control)	0	0	0
#F = vv (entire NS3 a.a. 1-618)	32	66	35
#E = vv (NS3 a.a. 1-452)	35	62	21
#G = vv (NS3 a.a. 1-182)	ND	0	18
#X = vv (NS3 a.a. 453-618)	7	4	1
Control Ag	8	0	1
Dengue-4 Ag	57	68	47

*Effector:target ratio was 8:1 for JK32, 15:1 for JK34 and 6:1 for JK43. 6 hours assay.

A-12. Mapping of the epitope recognized by JK34 using overlapping synthetic peptides

To further map the epitopes we synthesized 22 overlapping peptides which cover a.a. 183-452 of NS3 according to the amino acid sequence of NS3 of dengue-4 virus, Caribbean strain 814669. JK34 lysed LCL pulsed with a peptide #4 (a.a. 251-265) (Table 15). This result indicates that the epitope recognized by JK34 is located within a.a. 251-265 of NS3.

Table 15: Recognition of a.a. 251-265 by a dengue serotype-cross reactive CD4+ T cell clones, JK34*

Peptide	a.a. number on NS3	% specific ^{51}Cr release
1	183-197	3.7
2	191-205	5.1
3	213-227	2.2
4	251-265	26.1
5	258-272	5.4
6	266-280	3.3
7	273-287	2.4
8	281-295	0.6
9	288-302	6.3
10	296-310	4.2
11	303-317	4.0
12	311-325	5.1
13	333-347	7.0
14	356-370	4.0
15	378-392	3.8
16	386-400	5.8
17	401-415	5.5
18	408-422	3.2

19	416-430	3.7
20	423-437	5.8
21	431-445	6.4
22	438-452	3.8
No peptide D4V - Ag		2.5

*Autologous LCL were cultured with peptides at 20 uM for 20 hours, and used as target cells. Effector:target ratio was 8:1. 6 hour assay.

B. Human CD8+ T cell responses to dengue virus infection

B-1. Stimulation of dengue 4-immune PBMC with live or UV-inactivated dengue virus

PBMC taken from a dengue 4-immune donor were placed in culture with either live dengue virus or virus which had been exposed to UV light and shown to have no plaque-forming capacity. After 6 days, the cells were pulsed with ^{3}H thymidine and proliferation was assessed. The results in Table 16 show that the dengue 4-immune PBMC responded best to dengue 4. There was also a lower but significant response to dengue 2, which indicated that the response was serotype cross-reactive. Non-immune PBMC did not respond (data not shown), and UV-inactivated dengue 4 failed to generate a response.

Table 16: Stimulation of dengue 4-immune PBMC with live and UV-inactivated dengue virus

Exp.	Stimulus	^{3}H cpm	Stimulation index
1	Dengue 4	26,712	5.6
	Dengue 2	13,137	2.7
	Mock	4,789	-
2	Dengue 4	19,652	16.0
	Dengue 4 UV	627	0.5
	Mock	1,209	-

B-2. Lysis of dengue virus-infected fibroblasts by dengue 4-immune PBMC stimulated by live dengue virus

PBMC from a dengue 4-immune donor were tested for cytotoxic activity against dengue virus-infected autologous fibroblasts after 7-9 days of stimulation with dengue virus. Fibroblasts were chosen as target cells to detect $CD8^{+}$ CTL because they do not constitutively express class II MHC antigens. The results in Table 17 indicate that dengue 4-immune PBMC stimulated with either

dengue 2 or dengue 4 virus lysed fibroblasts infected with either serotype. Uninfected cells were also lysed, but the levels of lysis were always lower than those against virus-infected targets. These results show that the cytotoxic response is serotype cross-reactive.

Table 17: Lysis of dengue virus-infected fibroblasts by dengue 4-immune PBMC stimulated with dengue*

Exp.	PBL stimulated with:	% specific ^{51}Cr release from fibroblasts infected with: dengue 4	dengue 2	Mock
1	Dengue 4	26	26	2
	Dengue 2	28	24	7
	Mock	2	5	2
2	Dengue 4	62	58	2

*In exp. 1 the effector to target ratio was 40:1 in a 4 hour assay. In exp. 2 the effector to target ratio was 100:1 in a 4 hour assay.

B-3. Characterization of cytotoxic activity

Antibody blocking and antibody-C′ depletion studies were carried out to determine the identity of the killer cells. Table 18 shows that inclusion in the cytotoxicity assay of an antibody against class I antigens, W6/32, inhibited the killing of dengue 2-infected fibroblasts by 48%, which indicates that much of the killing was class I antigen-restricted. An antibody to class II DR antigens, OKIa-1, inhibited the killing poorly, showing that the killing was not DR-restricted. Since fibroblasts do not express any class II antigens under these conditions, killing restricted by the other class II (DP, DQ) can also be excluded.

Treatment of the effector population with anti-CD8 and C′ resulted in the death of 37% of these cells, and reduced their lytic capacity against dengue 2-infected fibroblasts by 61% (Table 18). Anti-CD4 and C′ treatment similarly killed 35% of the effector population, but failed to inhibit the cytotoxic activity (Table 18). Anti-Leu 11b and C′ treatment failed to induce either a significant decrease in viability (data not shown) or to reduce the cytotoxic capacity of effector cells (Table 18). Taken together, these results indicate that the effector cells in this system were virus-specific $CD8^+$ HLA class I-restricted serotype cross-reactive CTL.

Table 18: Characterization of cytotoxic activity*

Exp.	Dengue-4 stimulated PBMC treated with:	% specific ^{51}Cr release from dengue 2-infected fibroblasts	% inhibition
1	medium	65	-
	W6/32	34	48
	OKIa-1	55	15
2	C′	46	-
	anti-CD8 + C′	18	61
	anti-CD4 + C′	50	-9
	anti-Leu 11b + C′	44	4

*The effector to target ratio was 100:1 in a 4 hour assay.

B-4. Lysis of fibroblasts infected with dengue-vaccinia recombinants

We examined the proteins which are targets for dengue-specific class I-restricted CTL by infecting fibroblasts with dengue-vaccinia recombinant viruses, each of which contains a different portion of the dengue 4 genome. Recombinant A contains genes which code for dengue proteins NS1, NS2a, NS2b, NS3, NS4a, and 84% of NS4b. Recombinant B codes for C, pre-M, E, NS1 and NS2a. Recombinant C includes genes coding for NS1 and NS2a, and recombinant E codes for the E protein. Recombinant D is the parental vaccinia virus which contains the lac Z gene and has none of the dengue genome and serves as a control. Table 19 shows that CTL lysed fibroblasts infected with recombinants A, B and E, but not C and D. These results show that E is a target protein for these CTL, but NS1 and NS2a are not. The data also imply that at least one of the NS2b, NS3, NS4a, or NS4b proteins is a target for these CTL. From these studies, we cannot determine whether C, Pre-M, and NS5 contain target epitopes for CTL. Recombinant vaccinia viruses which contained the genes for the dengue 2 C, pre-M and E proteins and the genes for dengue 2 NS1, NS2a proteins were also used to infect autologous fibroblasts. Consistent with the concept that these CTL are cross-reactive, fibroblasts infected with the dengue 2 recombinant expressing C, pre-M, and E proteins were lysed by dengue 4 virus-stimulated, dengue 4-immune PBMC. However, these PBMC did not lyse fibroblasts infected with the dengue 2 recombinant expressing NS1 and NS2a (data not shown).

Table 19: Lysis of fibroblasts infected with dengue-vaccinia recombinants*

Fibroblasts infected with:	% specific ^{51}Cr release: Exp. 1	Exp. 2
Dengue 2	94	ND
Dengue 4	72	31
A=v[NS1, NS2a, NS2b, NS3, NS4a, NS4b]	39	17
B=v[C, pre-M, E, NS2, NS2a]	8	13
C=v[NS1, NS2a]	-2	0
D=vaccinia control	-	-
E=v[E]	13	28
Mock-infected	-4	-1

*Effectors were from dengue 4-immune PBMC stimulated for 8 days with dengue 4 virus. Assay length was 4 hours. E:T was 100:1 in Exp. 1 and 40:1 in Exp. 2. Lysis against targets infected with vaccinia virus (D=vaccinia control) was 12% in both experiments, which was subtracted for clarity.

B-5. Dengue virus-specific CD8+ CTL clones

To further characterize dengue virus-specific $CD8^+$ CTL, we established 16 $CD8^+$ CTL clones and lines from PBMC of a donor #C. These CTL clones lysed dengue-2 virus-infected autologous fibroblasts, but did not lyse uninfected fibroblasts (Table 20).

Table 20: Lysis of dengue-2 virus-infected autologous fibroblasts by $CD8^+$ CTL clones*

Lines and clones	Effector: Target Ratio	% specific 51CR release: Dengue-2 infected fibroblasts	Uninfected fibroblasts	K562
Long-term cultured lines				
a	3	21	1	0
b	5	10	0	0
c	10	40	0	ND
d	10	18	0	ND
e	10	51	5	ND
f	10	43	1	ND
Clones				
1.M	10	27	3	2
1.23	2	30	0	0
1.27	7	47	0	0
1.28	5	15	0	0
2.1	8	28	3	ND

2.3	9	8	0	ND
2.6	2	27	0	ND
2.7	2	26	0	ND
2.8	5	31	0	ND
2.9	1	27	0	ND

*6 hour assay.

Two of these CTL clones were further examined for virus and dengue serotype specificity. Clone #2.8 recognized dengue-2 and dengue-4 viruses, but did not recognize dengue-1, dengue-3, West Nile virus or yellow fever virus. Clone #2.9 recognized four serotypes of dengue viruses, but did not recognize West Nile virus or yellow fever virus (Table 21). Therefore, clone #2.8 is dengue subcomplex-specific and #2.9 is dengue serotype cross-reactive.

Table 21: Different virus- and dengue serotype-specificity of two $CD8^+$ CD4- clones*

	% specific 51CR release						
Clone	Dengue-1	Dengue-1	Dengue-3	Dengue-4	West Nile	Yellow Fever	Control Ag
#2.8	0	32	0	54	0	0	1
#2.9	77	88	96	100	0	0	0

*Autologous LCL were cultured with antigens for 20 hours and used as target cells. Effector:target cells were 5:1. 6 hour assay.

B-3. HLA restriction in the lysis of target cells by dengue virus-specific CD8+ CTL clones

HLA restriction in the lysis of target cells by these CTL clones were examined, using monoclonal antibodies to HLA class I, HLA DP, HLA DQ and HLA DR. Monoclonal antibodies to HLA class I (W6/32) inhibited the lysis of target cells by all the clones examined (Table 22). Therefore, these dengue virus-specific $CD8^+$ CTL clones are HLA class I-restricted.

Table 22: HLA class I-restriction in the lysis of target cells by dengue virus-specific $CD8^+$ CTL clones*

		% specific 51Cr release				
Target cells	Clones	No Antibody	Anti-HLA Class I	Anti-HLA DR	Anti-HLA DQ	Anti-HLA DP
Dengue 2-infected fibroblasts						
	1.27	26	6	14	18	20
	2.8	37	17	31	31	28
	a	43	2	32	ND	34
	e	51	13	49	ND	39

Dengue 2 Ag-cultured LCL					
2.8	71	2	70	77	71
Dengue 4 Ag-cultured LCL					
2.8	93	0	92	89	81

*Effector:target ratio was 10:1, 6 hour assay. W6/32, B7/21.7, S3/4 and OKIa1 were used as anti-HLA class I, anti-HLA DP, anti-HLA DQ and anti-HLA DR, respectively.

Clone #2.8 was further examined for HLA restriction, using allogenic target cells which partially share HLA class I antigens with autologous target cells (Table 23). Clone #2.8 lysed all the dengue 4 Ag-cultured allogeneic target cells which share HLA B35. These results indicate that clone #2.8 is HLA B35 restricted.

Table 23: HLA B35-restricted lysis of target cells by a clone #2.8

Target cells	HLA class I alleles A	B	C	% specific 51Cr release Dengue 4 Ag	Control Ag
Autologous (CA)	2,23	35,44	4,4	93	0
GM3106	1,1	35,35	4,4	80	9
GM3104A	3,3	35,35	4,4	78	0
JC	3,24	35,35	4,4	78	1
Thai 1975b	2,33	44,44	3,3	0	0
GM6816	2,2	38,38	?	0	0
IK	24,24	44,44	?	0	0
66390	3,24	7,62	4,7	0	0
GM06825A	23,23	7,7	7,7	0	0

We then used Hmy2CIR cells transfected with HLA B35 or HLA B51 as target cells to confirm our observations in previous experiments. Hmy2CIR cells do not express HLA A and HLA B alleles (Table 24). These cells were cultured with dengue 4 antigen and used as target cells. Clone #2.8 lysed HLA B35-transfected Hmy2CIR cells cultured with dengue-4 Ag, but did not lyse B-51 transfected Hmy2CIR cells cultured with dengue-4 Ag. This result confirmed our conclusion that clone #2.8 lyse target cells in a HLA B35-restricted fashion.

Table 24: Lysis of dengue 4 Ag-cultured, HLA B35-transfected target cells by clone #2.8

Effector:Target ratio	% 51CR release Hmy2CIR/B35	Hmy2CIR/B51
30	55	7
10	54	6
3	31	5
1	19	0

B-7. Mapping epitope recognized by dengue virus-specific CD8+ CTL clones

We are mapping epitopes recognized by dengue virus-specific $CD8^+$ CTL clones, using recombinant vaccinia viruses. Recombinant vaccinia virus #A contains dengue-4 genome which codes for NS1, NS2a, Ns2b, NS3, NS4a and NS4b proteins. Recombinant #B codes for the dengue-4 C, Pre-M, E, NS1 and NS2a. Recombinant vaccinia viruses #D, E#, #F, and #X were described above. Clone #2.8 lysed target cells infected with #A, #F and #X, and clone #2.9 lysed target cells infected with #F and #X. These results indicate that clones #2.8 and #2.9 recognize NS3, and that the epitopes which are recognized by #2.8 and #2.9 are located within a.a. 453-618 of the NS3 protein (Table 25).

Table 25: Lysis of autologous LCL infected with dengue-vaccinia recombinant viruses by $CD8^+$ CTL clones[a]

	% specific 51Cr release		
Target cells	Clone #8		
infected with	Exp. 1	Exp. 2	Clone #9
#A=VV[NS1, NS2a, NS2b, NS3, NS4a, NS4b]	29	ND	ND
#B=VV[C, pre-M, E, NS1, NS2a]	0	ND	ND
#D=vaccinia control	0	1	ND
#F=VV[whole NS3, a.a. 1-618]	28	34	14
#E=VV[NS3, a.a. 1-452]	0	0	0
EX=VV[NS3, a.a. 453-618]	ND	39	14

[a]Effector:target ratio was 10:1. 6 hour assay.

C. Activation of T lymphocytes in dengue virus infections

We examined levels of lymphokines (IL-2 and IFNγ) and soluble cell surface proteins (sIL-2, sCD4 and sCD8) released from activated T lymphocytes in unselected Thai children hospitalized with DHF. To determine whether differential cytokines or cellular immune responses were associated with hemorrhage and plasma leakage that are the pathognomonic feature of DHF, we also examined these same serum factors in patients who were infected with dengue virus but who did not have plasma leakage (Dengue fever). Children with DF were drawn from the same consecutive hospital case series as those with DHF, or they were drawn from a consecutive series of DF cases identified prospectively during a longitudinal study of illnesses leading to school absence in rural Thailand. To determine which findings in the entire set of patients with dengue virus infection were indicative of a host response to viral infection, we examined sera collected from healthy Thai children from the same cohort which yielded the dengue fever cases.

Table 26: Age and sex distribution, and serological data of the donors

Donor	Number	Sex Male	Female	Average age ± S.D. (Range)	Dengue serology Primary	Secondary
DHF	59	28	31	8.9 ± 3.0 (4-14)	7	52
DF	41	22	19	9.8 ± 2.1 (5-14)	6	35
Uncharacterized febrile diseases	26	12	14	9.5 ± 1.6 (6-14)	0	0
Healthy children	97	45	52	7.9 ± 1.6 (6-13)	0	0

C-1. Levels of sIL-2R, sCD4 and sCD8 in patients with DHF or DF

The sera from patients with DHF or DF were examined for the levels of sIL-2R, sCD4 and sCD8, and the levels were compared with those in the sera of healthy Thai children.

The levels of sIL-2R were significantly higher in the sera of patients with DHF and in the sera of patients with DF than in the sera of healthy Thai children during the examined period ($p<0.001$ for DHF, $p<0.001$ for DF) (Figure 3).

The levels of sCD4 were higher in the sera of patients with DHF on days 3-8 after onset of fever ($p<0.001$) than in the healthy children. The levels of sCD4 in the sera of patients with DF were higher than the levels in healthy children on day 1-2 after onset of fever ($p<0.05$), but not on day 3-4 (Figure 4).

The levels of sCD8 were higher in the sera of patients with DHF on days 3-20 after onset of fever than in the sera of healthy children ($p<0.001$). The levels of sCD8 in the sera of patients with DF were not elevated during days 1-20 (Figure 5). These results suggest that $CD4^+$ T lymphocytes are activated in vivo during DHF and DF, and that activation of $CD8^+$ lymphocytes is evident in DHF, but not in DF.

Figure 3. Levels of soluble IL-2 receptor in the acute sera of patients with DF or DHF.

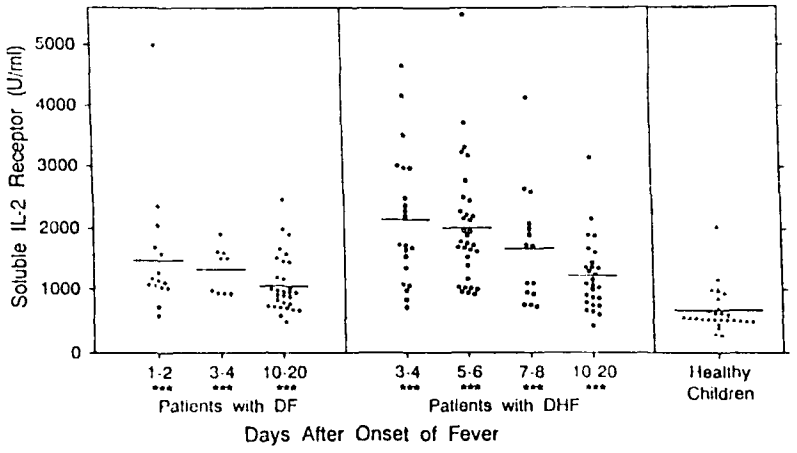


Figure 4. Levels of soluble CD4 in the acute sera of patients with DF or DHF.

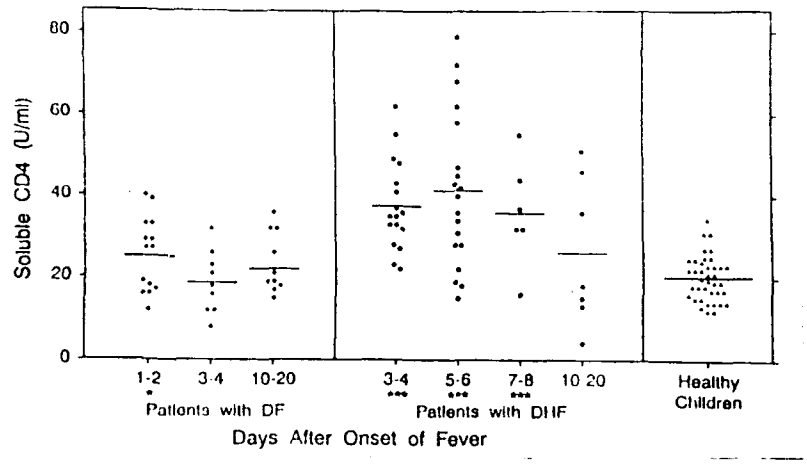
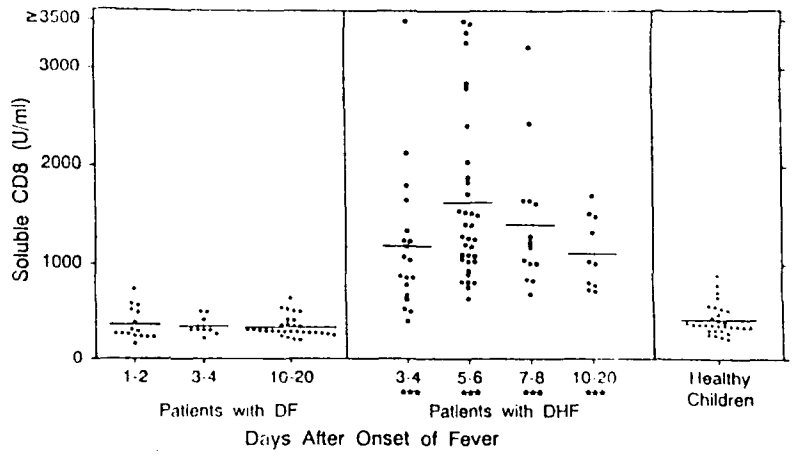


Figure 5. Levels of soluble CD8 in the acute sera of patients with DF or DHF.



C-2. Levels of IL-2 and IFNγ in the sera of patients with DHF or DF

The sera from patients with DHF or DF were examined for IL-2 or IFNγ. IL-2 levels greater than 10 U/ml were detected in 63% (26/41) of patients with DHF during days 3-8 after onset of fever, and in 69% (18/26) of patients with DF during days 1-4, while only 2 of 28 sera of healthy children contained IL-2 greater than 10 U/ml ($p<0.001$ for DHF, $p<0.001$ for DF by Chi square test) (Figure 6). The titers of IL-2 were significantly higher in the sera of patients with DHF ($p<0.001$ on days 3-8, 10-20) and in the sera of patients with DF ($p<0.001$ on days 1-2 and 10-20, and $p<0.01$ on days 3-4) than in the sera of healthy Thai children (Figure 6).

IFNγ was detected in 97% (34/35) of the patients with DHF, and in 91% (31/34) of patients with DF, while it was detected in 13% (4/30) of the sera of healthy children ($p<0.001$ for DHF, $p<0.001$ for DF by Chi-square test) (Figure 7). The titers of IFNγ in the sera of patients with DHF on day 3-8 ($p<0.001$ on days 3-6 and $p<0.02$ on days 7-8) and in the sera of patients with DF on days 1-4 ($p<0.001$) were significantly higher than those in the sera of healthy children (Figure 7). These results are consistent with those in Figures 1-3, and indicate that T lymphocytes are activated in vivo during DHF and DF.

Figure 6. Levels of IL-2 in the acute sera of patients with DF or DHF.

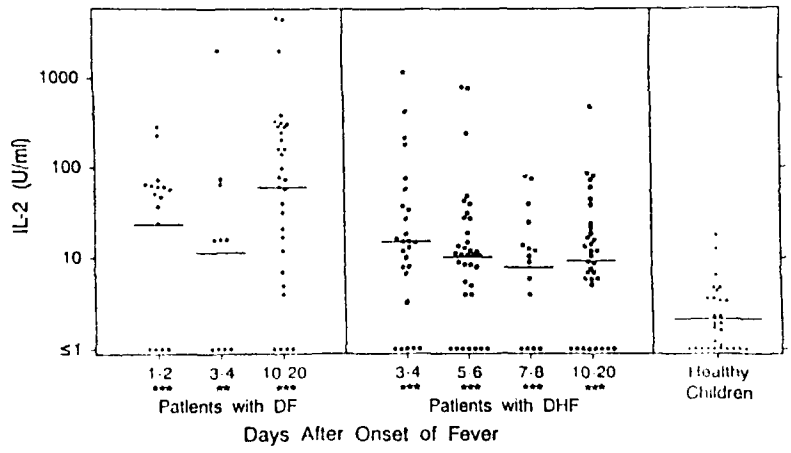
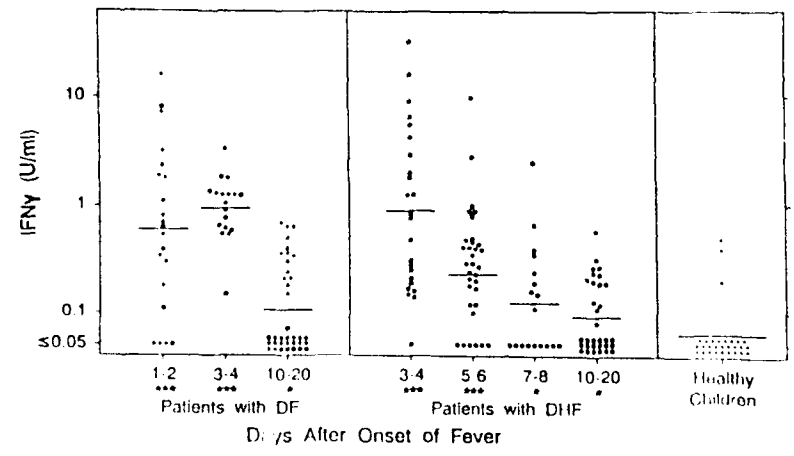


Figure 7. Levels of IFNγ in the acute sera of patients with DF or DHF.



C-3. Comparison of the levels of soluble cell surface proteins and lymphokines between DHF and DF

A comparison of sIL-2R, sCD4, sCD8, IL-2 and IFNγ among patients with DHF or DF were made for sera collected on illness days 3-4, because specimens were obtained from patients with DHF during days 3-8 after onset of fever and from patients with DF during days 1-4. The sera of patients with DHF contained higher levels of sIL-2R ($p<0.05$), sCD4 ($p<0.001$), and sCD8 ($p<0.001$) than the sera of patients with DF, although the levels of IL-2 and IFNγ were not different between DHF and DF (Table 27). These results suggest that activation of T lymphocytes is greater in DHF than in DF. The levels of sIL-2R, IL-2 and IFNγ were elevated in the sera of patients with unidentified febrile diseases on days 1-2, and the levels are similar to those in the sera of patients with DF (data not presented).

Table 27: Levels of soluble cell surface proteins and lymphokines in DHF and DF on day 3-4 after the onset of fever*

Markers	DHF Titers (U/ml) (No. of samples)	DF Titers (U/ml) (No. of samples)	p value
SIL-2R	2232±218 (23)	1325±127 (9)	<0.05
SCD4	37.6±2.7 (17)	18.7±2.7 (9)	<0.001
SCD8	1188±176 (19)	344±33 (9)	<0.001
IL-2 (Log_{10})	1.181±0.156 (27)	1.062±0.348 (10)	NS
IFNγ (Log_{10})	-0.044±0.153 (24)	-0.024±0.068 (18)	NS

*Levels of sIL-2R, sCD4, sCD8, IL-2, IFNγ on day 3-4 after the onset of fever were compared between DHF and DF by Student's t test. The titers of IL-2 and IFNγ were log-transformed for analysis. The numbers in the parentheses depict the number of samples. NS denotes statistically not significant.

C-4. Levels of sIL-2R, sCD4, sCD8, IL-2 and IFNγ in patients with DHF before and after the day of defervescence

The timing of plasma leakage in patients with DHF is quite predetectable; circulatory collapse occurs or peaks as fever subsides. Therefore, we examined the change in the levels of soluble cell surface proteins and lymphokines in patients with DHF around the day of defervescence termed day 0 in this analysis. Mean levels of sIL-2R, sCD4 and sCD8 were similar during day -1 to day 1 (Figure 8). The titers of IL-2 and IFNγ were highest one day before defervescence (Figure 9). These results suggest that activation of T lymphocytes reaches the peak as plasma leakage begins but before circulatory collapse becomes manifest.

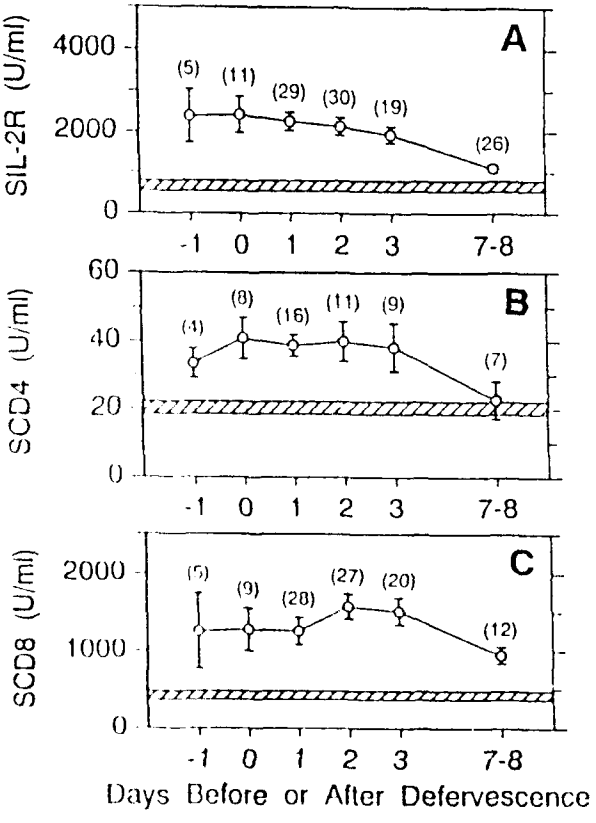


Figure 8. Levels of sIL-2R, sCD4, and sCD8 in patients with DHF before and after the day of defervescence. ▨: Mean titers ± 1.96 x SEM in the sera of healthy Thai children.

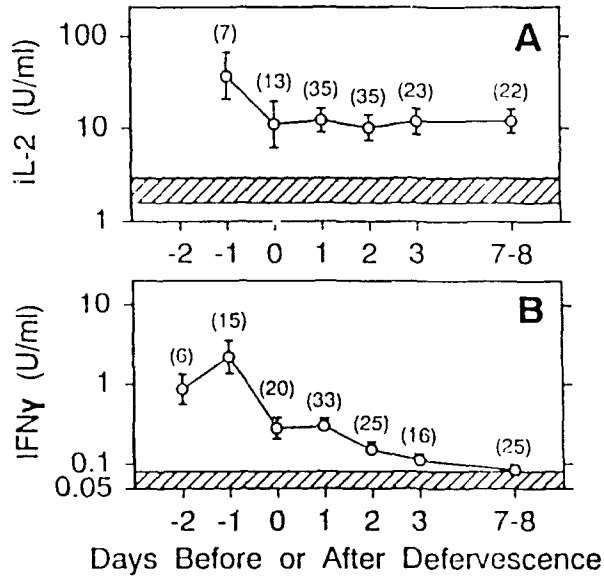


Figure 9. Levels of IL-2 and IFNγ in patients with DHF before and after the day of defervescence. ▨: Mean titer ± 1.96 x SEM in the sera of healthy Thai children.

C-5. Comparison of the levels of each serum factor among the WHO grades 1, 2 and 3 of DHF

The levels of soluble cell surface proteins and lymphokines were compared among grades 1, 2 and 3 of DHF from day -1 to day 3. Statistically significant differences were observed only in the levels of sCD4 between grade 1 and grade 3 one day after defervescence (Figure 10) and in the levels of IL-2 between grade 2 and grade 3 one day before defervescence (Figure 11). As a whole, the levels of sIL-2R, sCD4, sCD8, IL-2 and IFNγ were not different among grades 1, 2 and 3 of DHF. These results suggest that the degree of T cell activation is similar among grades 1, 2 and 3 of DHF, and that our grouping all grades of DHF for analysis was appropriate.

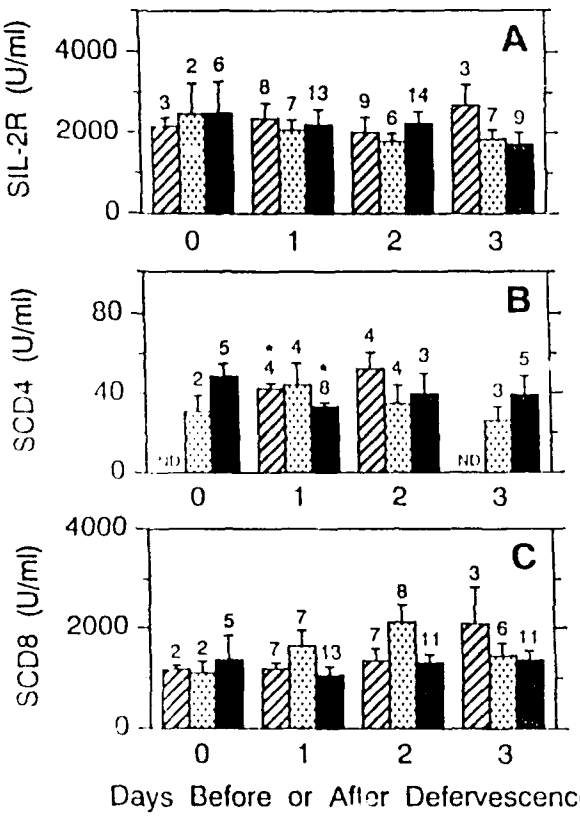


Figure 10. Levels of sIL-2R, sCD4 and sCD8 in grades 1, 2 and of DHF. Hatched bar, grade 1; dotted bar, grade 2; black bar, grade 3.

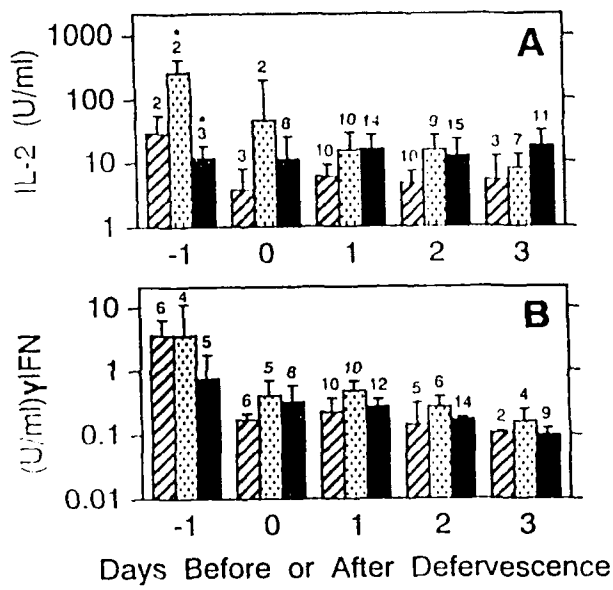


Figure 11. Levels of IL-2 and IFNγ in grades 1, 2, and 3 of DHF. Hatched bar, grade 1; dotted bar, grade 2; black bar grade 3.

D. Dengue virus infection of human cell lines

It is speculated that following feeding by mosquitos, the virus enter blood stream via lymphatics (10) and peripheral blood mononuclear cells (PBMC), especially monocytes, support virus infections (2). The study of dengue virus-human cell interaction is important to understand the pathogenesis of dengue virus infections. Many human cell lines are available; therefore, we decided to examine a variety of human cell lines to determine whether they could be infected with dengue virus and be useful for future studies.

D-1. Acute infection of human mononuclear cell lines with dengue-2 virus

Ten myelomonocytic cell lines, eight B cell lines and five T cell lines were used in the experiments. All the cell lines could be infected with dengue-2 virus in the absence of antibody (Table 28). Antibody to dengue-2 virus augmented dengue-2 virus infection of myelomonocytic cell lines determined by IF and virus assays. However, antibody did not augment infection of B or T cell lines. In myelomonocytic cell lines K562, HEL92-1-7, JOSK-I and JOSK-M cells contained a high percentage of antigen-positive cells, while HL-60, KG-1 and THP-1 contained fewer antigen-positive cells. In B cell lines Jiyoye, ARH-77 and IM-9 contained a high percentage of antigen-positive cells, while Ramos, Daudi and CA46 contained fewer antigen-positive cells. In T cell lines Jurkat and CEM contained a high percentage of antigen-positive cells.

Table 28: Dengue virus infection of human mononuclear cell lines*

		% dengue-2 antigen-positive cells			Virus titer (p.f.u./ml)
Cell line	Antibody[2]	24 hrs	48 hrs	72 hrs	at 48 hrs.
Myelomonocytic cell lines					
K562	+	90.8	95.9	99.0	5.0×10^6
	-	57.8	76.5	99.0	4.5×10^6
HEL92-1-7	+	35.8	35.4	41.8	2.3×10^5
	-	22.8	26.5	37.2	1.5×10^5
JOSK-I	+	37.5	34.3	18.5	5.0×10^5
	-	7.7	4.1	1.7	5.7×10^4
JOSK-M	+	28.6	23.0	8.4	2.3×10^5
	-	16.0	11.0	3.5	2.3×10^5
JOSK-S	+	16.1	9.5	6.4	1.6×10^5
	-	6.3	3.8	3.4	1.5×10^5
JOSK-K	+	15.1	14.1	3.9	4.0×10^5
	-	3.6	3.7	3.3	1.6×10^5
U937	+	15.7	17.4	4.1	3.8×10^4
	-	0.6	1.1	0.9	4.2×10^3
THP-1	+	7.5	6.8	9.8	1.1×10^5
	-	0.4	0.5	0.5	2.0×10^4

KG-1	+	2.4	1.7	0.9	1.3×10^4
	-	1.4	1.3	0.5	3.0×10^3
HL-60	+	1.7	2.9	1.6	1.4×10^3
	-	0.2	0.4	0.3	3.5×10^2
B cell lines					
Jiyoye	+	82.6	81.4	81.7	4.0×10^4
	-	82.4	86.4	85.0	5.0×10^4
ARH-77	+	28.4	45.7	30.8	5.4×10^5
	-	25.0	30.9	31.4	3.9×10^5
IM-9	+	22.6	22.7	17.2	3.5×10^5
	-	30.3	25.3	13.1	3.1×10^5
Raji	+	10.2	6.6	11.7	8.5×10^3
	-	7.8	9.6	13.7	9.5×10^3
HS-Sultan	+	8.8	6.9	8.3	4.0×10^3
	-	4.8	5.6	7.5	3.0×10^3
CA46	+	1.5	1.5	1.3	4.0×10^3
	-	2.5	2.2	1.0	2.0×10^3
Daudi	+	1.1	1.1	0.8	1.0×10^2
	-	0.5	1.8	0.8	1.0×10^2
Ramos	+	0.8	0.5	0.4	8.0×10^0
	-	0.5	0.5	0.3	5.0×10^0
T cell lines					
Jurkat	+	50.0	39.2	39.7	6.6×10^4
	-	48.7	44.5	34.5	6.1×10^4
CEM	+	36.3	27.0	21.3	1.3×10^5
	-	34.0	23.4	18.5	1.5×10^5
HSB-2	+	13.8	24.4	81.7	1.0×10^5
	-	19.5	28.5	84.0	3.0×10^5
Molt 4	+	8.2	16.8	41.7	1.1×10^5
	-	7.6	15.3	44.4	1.7×10^5
Molt 3	+	4.0	12.9	14.0	7.5×10^3
	-	8.1	12.0	21.3	1.3×10^4

[a]Cells were infected with dengue-2 virus at a m.o.i. of 5 p.f.u./cell in the presence or absence of antibody as described in Materials and Methods.

[b]Anti-dengue-2 antibody was used at final dilution of 1:10,000. + represents presence of antibody and - represents absence of antibody.

D-2. Establishment of cell lines persistently infected with dengue-2 virus

We determined the ability of certain cells to become persistently infected with dengue-2 virus. K562, Raji and HSB-2 cell lines were infected with dengue-2 virus at a m.o.i. of 5 p.f.u./cell in the absence of antibody to dengue-2 virus, and were cultured for 25 weeks. Cells were resuspended at a concentration of 2×10^5/ml twice a week. The percent of dengue-2 virus antigen-positive cells was almost 100% one week after infection of K562 (Figure 12) and HSB-2 cells (Figure 13), and three weeks after infection of Raji cells (Figure 14). A high percentage (more than 70%) of dengue-2 virus antigen-positive cells was observed for 25 weeks. These results demonstrate that persistent dengue-2 virus infections were readily established in myelomonocytic, B and T cell lines.

We measured dengue-2 virus titers in culture supernatant fluids 22 weeks after the infection. Infectious dengue-2 virus at the titer of 2.6×10^2 p.f.u./ml, 1.8×10^2 p.f.u./ml and 1.0×10^2 p.f.u./ml were detected in the supernatant fluids of persistently infected K562, HSB-2 and Raji, respectively. Intracellular dengue virus was then detected after three freeze-thaw cycles of 1×10^6 infected cells. Intracellular virus titers were 2.2×10^2 p.f.u./ml and 4.0×10^1 p.f.u./ml in K562 and HSB-2 cells, respectively. Intracellular virus was not detected (<10 p.f.u./ml) in persistently infected Raji cells.

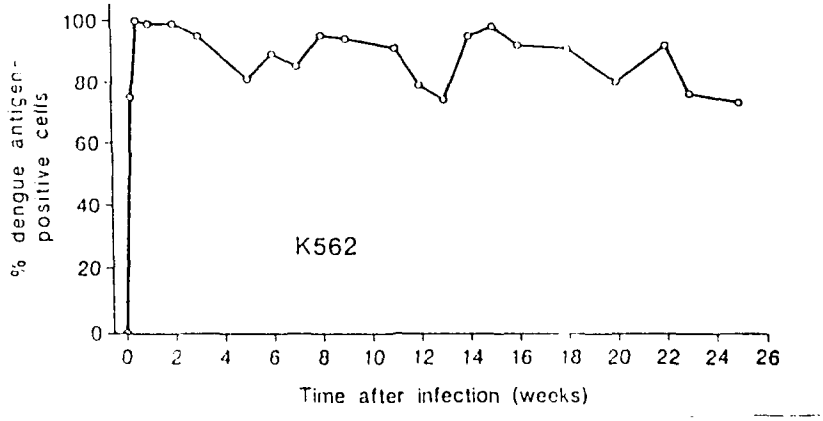


Figure 12. Persistent infection of K562 cells infected with dengue-2 virus at an m.o.i of 5 p.f.u./cell. Percentage of dengue-2 virus antigen-positive cells was determined using IF staining.

Figure 13. Persistent infection of HSB-2 cells infected with dengue-2 virus at an m.o.i. of 5 p.f.u./cell. Percentage of dengue-2 virus antigen-positive cells was determined using IF.

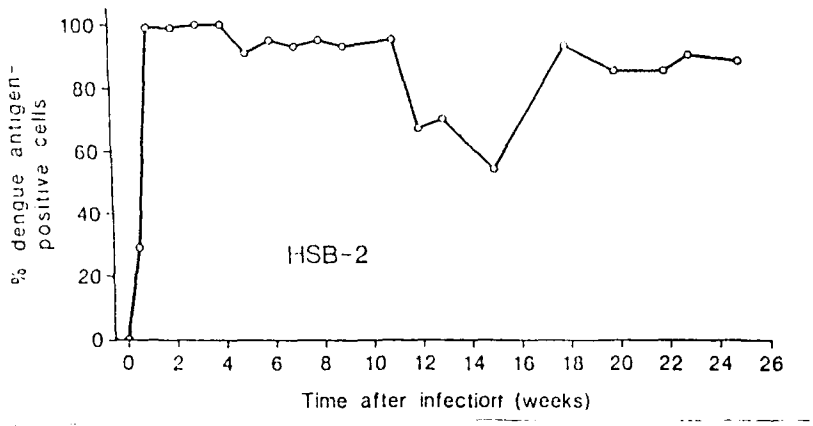
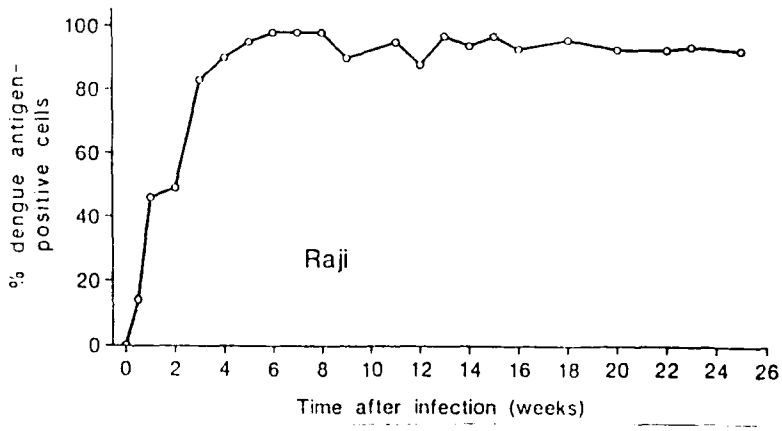


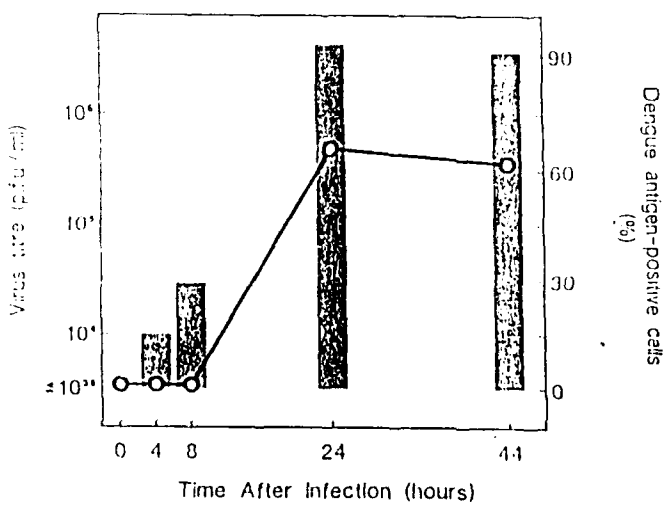
Figure 14. Persistent infection of Raji cells infected with dengue-2 virus at an m.o.i. of 5 p.f.u./cell. Percentage of dengue-2 virus antigen-positive cell was determined using IF.



D-3. Infection of human fibroblasts with dengue-2 virus

Human skin fibroblasts were infected with dengue-2 virus, and the percentage of dengue virus antigen-positive cells and the infectious virus titers in the culture supernatant fluids were examined. Dengue virus antigen-positive cells were detected as early as 4 hours after infection and the percentage of dengue virus antigen-positive cells reached maximum levels by 24 hours after infection (Figure 15). High titers of infectious dengue virus were detected at 24 and 44 hours after infection. These results indicate that fibroblasts can be infected with dengue virus.

Figure 15. Time course of the appearance of infectious dengue virus in the culture supernatant fluid and cytoplasmic dengue virus antigens in infected fibroblasts.
○: Titers of infectious dengue virus culture fluids.
▨: The percentage of dengue virus antigen-positive cells.



D-4. Infection of fibroblasts with other dengue viruses, YFV and WNV

To determine whether infection of human skin fibroblasts is a common feature of flaviviruses, we used 7 strains of other mosquito-borne flaviviruses, including dengue-1, dengue-3, dengue-4, YFV and WNV in addition to dengue-2 virus, New Guinea C strain (Table 29). Fibroblasts were infected with viruses at an m.o.i. of 10 p.f.u./ml, and the percentage of antigen-positive cells were examined by IF staining using a flavivirus-crossreactive monoclonal antibody 4G2. All 7 viruses examined infected fibroblasts, although the percentage of antigen-positive cells varied depending on the virus strains. These results suggest that most, if not all, strains of mosquito-borne flaviviruses can infect human skin fibroblasts.

Table 29: Infection of human skin fibroblasts with dengue, yellow fever and West Nile viruses*

Viruses	Strain	% antigen-positive cells
Dengue-1	Hawaii	40
Dengue-2	New Guinea C	78
Dengue-3	CH53489	53
Dengue-3	904	32
Dengue-4	814669	17
Dengue-4	Thai 286	55
Yellow fever virus	17D	17
West Nile virus	E101	59

*Human skin fibroblasts were infected with each virus at an m.o.i. of 10 p.f.u./cell. The percentage of antigen-positive cells was examined 24 hours after infection by immunofluorescence staining using a flavivirus-crossreactive monoclonal antibody 4G2.

E. Enhancement of dengue virus infection by antibody (antibody-dependent enhancement)

Enhancement of dengue virus infection by antibodies was first reported by Halstead et al in 1977 (11,12). They reported that human sera from dengue virus-infected patients, monkey sera from dengue virus-immune monkeys, hyperimmune mouse and rabbit sera to dengue virus enhanced dengue virus infection of peripheral blood mononuclear cells from non-immune humans and monkeys when the cells were infected with dengue virus in the presence of these anti-dengue virus sera. These sera enhanced infection at a dilution where they lost neutralizing activity. It is understood today that dengue virions and IgG antibody to dengue virus forms virus-antibody complex, and that binding of dengue virus-antibody complexes to Fcγ receptors via the Fc portion of IgG results in augmentation of dengue virus infection. Antibody-dependent enhancement, therefore, results from interaction of three components; dengue virus, antibody and Fcγ receptor.

IgG-Fc receptors play a key role in antibody-dependent enhancement. Three types of human FcγR have been identified (13-15). FcγRI is a 72-Kda molecule designated CD64 which is detected exclusively on monocytes/macrophages and IFNγ-treated granulocytes. FcγRI binds human monomeric IgG with high avidity ($Ka=10^8-10^9\ M^{-1}$), and with an isotype specificity of IgG1=IgG3>IgG4>>IgG2. FcγRII is a 40-Kda molecule designated CDw32 which is detected on monocytes, neutrophils, eosinophiles, platelets and B cells. FcγRII is a low avidity receptor and binding of monomeric IgG could not be measured. However, the presence of FcγRII has been demonstrated by the binding of immune

complexes, and FcγRII binds human IgG with a subclass specificity of IgG1=IgG3>>IgG2, IgG4.

E-1. Antibody-dependent enhancement of dengue virus infection mediated by FcγRII

K562 cells were examined for the expression of FcγRI, FcγRII and FcγRIII by quantitative flow cytometry using specific monoclonal antibodies (mAb) 32.2, IV.3 and anti-Leu11a, respectively. K562 cells expressed high levels of FcγRII, but did not express FcγRI or FcγRIII. U937 cells expressed FcγRI and FcγRII, but did not express FcγRIII. The K562 cell line was selected for defining the role of FcγRII in dengue virus infections because it expresses only FcγRII.

K562 cells were infected with dengue virus in the presence or absence of anti-dengue-2 mouse sera at a final dilution of $1:10^4$, and the percentage of dengue virus antigen-positive cells were examined 24 and 48 hours after infection. A dose response study showed that anti-dengue-2 serum enhanced infection best when used at a final dilution of $1:10^4$ (data not presented). K562 cells which were infected with dengue virus in the presence of the diluted polyclonal preparation of anti-dengue virus antibody contained a significantly higher percentage of antigen-positive cells than those infected in the absence of antibody (Table 30). Normal mouse serum, which does not contain anti-dengue virus antibodies, did not enhance infection at any dilutions ranging from 1:10 to $1:10^7$. These results suggest that anti-dengue virus antibodies enhance dengue virus infection by binding to Fc RII after the formation of virus-antibody complexes.

We examined whether infection of K562 cells with virus-antibody complex results in viral production. K562 cells were treated with trypsin to inhibit infection by dengue virus which did not make virus-antibody complex as previously reported (17,18), and infected with dengue virus with or without antibody at 4°C as described in the Materials and Methods. K562 cells infected with dengue virus in the presence of antibody produced higher tiers of virus than K562 cells infected with virus alone (4.5×10^4 p.f.u./ml with antibody and 1.2×10^4 p.f.u./ml without antibody in exp. 1, and 1×10^4 p.f.u./ml with antibody and 3×10^3 p.f.u./ml without antibody in exp. 2). These results suggest that infection with virus-antibody complex results in viral production.

Table 30: Augmentation of dengue virus infection of K562 cells by anti-dengue virus antibody*

Exp.	m.o.i. (p.f.u./cell)	Incubation time (hours)	% of dengue antibody-positive cells** With anti-dengue antibody	No antibody
1	0.125	24	26.3[a]	18.0
2	0.125	24	18.4[b]	12.7
3	0.03	48	12.8[c]	5.6
4	0.125	48	20.8[d]	11.0
5	1.0	72	57.0[e]	36.6

*K562 cells were infected with dengue virus in the presence or absence of anti-dengue-2 mouse sera at a final dilution of $1:10^4$. The percentage of dengue virus antigen-positive cells were determined by immunofluorescence staining.

**The percentage of antigen-positive cells was compared between the cells infected in the presence of anti-dengue antibody and those infected in the absence of antibody by Chi square test.

[a] $p<0.05$, [b] $p<0.05$, [c] $p<0.02$, [d] $p<0.01$, [e] $p<0.001$

E-2. Inhibition of anti-dengue virus antibody-mediated infection by a monoclonal antibody specific for FcγRII

MAb IV.3, which is specific for FcγRII and is known to inhibit the binding of antigen-antibody complexes to FcγRII (16,17) was used to prove that enhancement of dengue virus infection by anti-dengue virus antibody is FcγRII-mediated.

K562 cells were incubated with mAb IV.3 and were then infected with dengue virus in the presence or absence of anti-dengue antibody. MAb IV.3 inhibited infection when cells were infected in the presence of anti-dengue antibody, but not when cells were infected in the absence of antibody. MAb 32.2, which is specific for FcγRI, did not inhibit infection in the presence or absence of anti-dengue virus antibody (Table 31, Exp. 1).

The Fab fragment of MAb IV.3 was then used to pretreat K562 cells prior to infection with dengue virus. The Fab of IV.3 also inhibited infection by virus-antibody complex, but did not inhibit infection by dengue virus alone (Table 31, Exp. 2); whereas, the $F(ab')_2$ of 32.2 did not affect infection. These results indicate that enhancement of infection by anti-dengue virus antibody is FcγRII-mediated.

Table 31: Anti-Fc RII monoclonal antibody inhibits antibody-mediated enhancement of dengue virus infection of K562 cells[a]

	Anti-Fc R antibodies	% dengue antigen-positive cells With anti-dengue antibody	No antibody
Exp 1	None	40.6[d]	31.5[d]
	IV.3	21.2[b]	31.3[c]
	32.2	40.9[c]	32.3[c]
Exp 2	None	28.6[e]	21.8[e]
	IV.3 (Fab)	16.7[b]	17.7[c]
	32.2 ($F(ab')_2$)	25.5[c]	21.7[c]

[a]K562 cells were incubated with monoclonal Ab IV.3 or 32.2 at a concentration of 100 ug/ml at 4°C for 1 hour. Cells were washed once and infected with dengue virus or dengue virus-antibody complex at an m.o.i. of 0.125 p.f.u./cell. The percentage of dengue virus antigen-positive cells was examined using immunofluorescence staining 24 hours after infection.

[b]$p<0.001$ compared with the cells not pretreated with anti-Fc R antibodies by Chi square test.

[c]$p>0.2$ (not significant) compared with the cells not pre-treated with anti-Fc R antibodies by Chi square test.

[d]The percentage of dengue virus antigen-positive cells was compared by Chi square test between the cells infected in the presence of anti-dengue virus antibody and those infected in the absence of anti-dengue virus antibody. $p<0.01$.

[e]Same as d. $p<0.05$.

E-3. Interferon gamma augments $Fc_{\gamma}R$-mediated dengue virus infection of human monocytic cells

U937 cells were incubated with 100 U/ml of recombinant IFN-γ (rIFN-γ) for 24 hours, and then were infected with dengue virus at a m.o.i. of 5 p.f.u./cell in the presence of anti-dengue mouse serum. The percentage of dengue antigen-positive cells was determined by indirect immunofluorescence staining 24 hours after infection. Anti-dengue serum at final dilutions of $1:10^3$, 10^4, and 10^5 augmented infection of nontreated U937 cells, and further augmented dengue virus infection, when U937 cells were pretreated with rIFN-γ (Figure 16). Normal mouse serum which did not contain detectable levels of anti-dengue antibody did not augment dengue virus infection of the nontreated or the IFN-γ-treated U937 cells (data not presented). Based on these results, we decided to use anti-dengue serum at a final dilution of $1:10^4$ in the following experiments.

U937 cells were incubated with variable amounts of rIFN-γ for 24 hours, and infected with dengue virus at a m.o.i. of 5 p.f.u./cell in the presence of the anti-dengue mouse serum at a final dilution of $1:10^4$. Pretreatment of U937 cells with rIRN-γ at concentrations from 1 to 10,000 U/ml increased the percentage of dengue antigen-positive cells. The percentage of antigen-positive cells reached a maximum level by pretreatment with IFN-γ at 100 U/ml (Figure 17). When U937 cells were infected with dengue virus in the absence of anti-dengue antibody, pretreatment with rIFN-γ did not increase the percentage of antigen-positive cells (1-3% without rIFNγ-treatment and 1-4% with rIFNγ-treatment).

Figure 16. Effect of dilution of anti-dengue serum on dengue virus infection of U937 cells. U937 cells were incubated with or without 100 of IFNγ per ml for 24 hours and infected with dengue virus at an m.o.i. of 5 p.f.u./cell in the presence of variable dilution of anti-dengue mouse serum. ○: U937 cells treated with IFNγ. ●: Nontreated IFN .

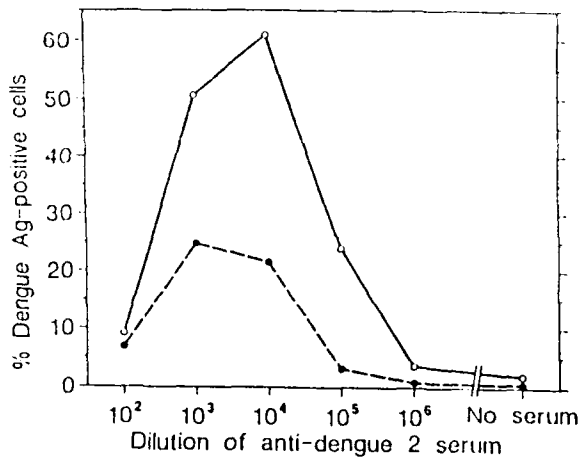
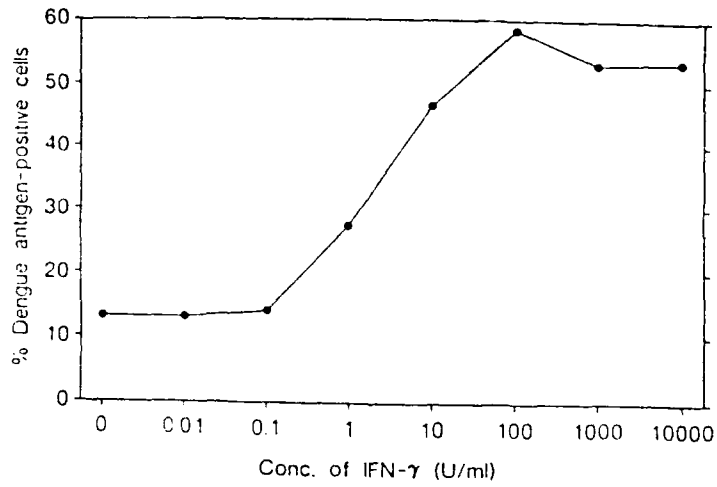


Figure 17. Dengue virus infection of U937 cells pretreated with IFNγ. U937 cells were incubated with variable concentrations of IFNγ for 24 hours and then infected with dengue virus-antibody complex at an m.o.i. of 5 p.f.u./cell.



Pretreatment of U937 cells with 100 U/ml of rIFN-γ also increased dengue virus titers detected in the culture fluids when cells were infected with virus in the presence of antibody, but not when cells were infected in the absence of antibody (Table 32).

Table 32: Virus titers in the culture fluids of dengue-infected U937 cells pretreated with IFN-γ*

	Virus titer (p.f.u./ml)	
IFN-γ (U/ml)	Infection in the presence of anti-dengue antibody	Infection in the absence of antibody
0	5.0×10^3	3.5×10^2
1	4.5×10^4	4.0×10^2
10	6.0×10^4	6.5×10^2
100	9.0×10^4	7.0×10^2
1000	1.0×10^5	6.0×10^2

*U937 cells were incubated with variable concentrations of rIFN-γ for 24 hours and infected with dengue virus at a m.o.i. of 5 p.f.u./cell in the presence of anti-dengue mouse serum at final dilution of 10^{-4}, or in the absence of antiserum. Cells were cultured at 2×10^5/ml in RPMI containing 10% FCS for 24 hours. Dengue virus contained in the culture fluids was titrated using a plaque titration assay.

E-4. Anti-IFN-γ antibody inhibits IFN-γ-induced augmentation of dengue virus infections

To confirm that IFN-γ contained in the culture fluid is responsible for the augmented dengue virus infection shown in Table 2, the culture fluid which contained 10 U/ml of IFN-γ was incubated with a monoclonal anti-IFN-γ antibody, and then was used to treat U937 cells. The culture fluid pretreated with an anti-IFN- antibody did not augment dengue virus infection but the culture fluid pretreated an anti-IFN-γ antibody did augment (Table 33). We also tried to block the effect or rIFN-γ using a monoclonal anti-IFN-γ antibody to confirm that rIFN-γ-induced augmentation of infection was due to IFN-γ, and was not due to other substances derived from the production of this recombinant IFN-γ in E. coli. U937 cells were pretreated with 10 U/ml of rIFN-γ which had been incubated with a monoclonal IFN- antibody or a polyclonal anti-IFN antibody. An anti-IFN-γ antibody inhibited the augmenting effect of rIFN-γ, but anti-IFN- antibody had no effect (data not presented). These results confirm that IFN-γ is responsible for augmentation of dengue virus infection shown in Table 32 and Figure 16 and 17.

Table 33: IFN-γ contained in the culture fluid of PBMC is responsible for augmenting dengue virus infection of U937 cells

Source of IFN-γ	Antibodies[c]	Percentage of dengue antigen-positive cells[d]
Dengue culture fluid[a]	None	66.0
	Anti-IFN-γ	20.5
	Anti-IFN-α	59.6
Control culture fluid[b]	None	20.9
None	None	15.0
	Anti-IFN-γ	16.4
	Anti-IFN-α	12.1

[a]Dengue culture fluid was obtained as described in the footnote of Table 2, and was diluted to contain 10 U/ml of IFN-γ.

[b]PBMC of the same donor were cultured with a control antigen for 7 days, and a culture fluid was collected. This culture fluid, which contained no detectable IFN-γ or IFN-α, was diluted similarly.

[c]Dengue culture fluid which contains IFN-γ was diluted to 10 U/ml and was then incubated with 1000 U/ml of monoclonal anti-IFN-γ and 2000 U/ml of anti-IFN-α at 4°C for 2 hours.

[d]U937 cells were incubated with culture fluids for 24 hours, and infected with dengue virus-antibody complexes at a m.o.i. of 5 p.f.u./cell. The percentage of dengue antigen-positive cells was determined by indirect immunofluorescent staining 24 hours after infection.

E-5. Human gamma globulin blocks IFN-γ-induced augmentation of dengue virus infection

It has been reported that IFN-γ increases Fcγ receptors on U937 cells (18,19). We tried to determine whether the IFN-γ-induced augmentation of dengue virus infection was Fcγ receptor mediated. U937 cells which had been treated with rIFN-γ at 1000 U/ml for 24 hours were incubated with γ-globulin at 4°C for 20 minutes. Cells were then infected with dengue virus-antibody complexes. γ-globulin inhibited infection by dengue virus antibody complexes of U937 cells which were pretreated with IFN-γ, wnereas bovine serum albumin at the same concentration had no effect. These results suggest that the IFN-γ induced augmentation of dengue virus infection is mediated by Fcγ receptors.

E-6. IFN-γ does not augment dengue virus infection of U937 cells in the presence of $F(ab')_2$ fraction of anti-dengue IgG antibody

We then used the $F(ab')_2$ fraction of anti-dengue IgG, to confirm that IFN-γ- induced augmentation of dengue virus infection is Fc receptor-mediated. Pretreatment of U937 cells with IFN-γ did not augment infection when cells were infected with dengue virus in the presence of $F(ab')_2$ prepared from anti-dengue IgG, but IFN-γ-pretreatment augmented infection when cells were infected with virus in the presence of purified anti-dengue IgG at 0.1 to 10 ug/ml (Table 34). This result confirms that the IFN-γ-induced augmentation of dengue virus infection is mediated by Fcγ receptors on U937 cells.

Table 34: $F(ab')_2$ prepared from anti-dengue IgG does not augment dengue virus infection of U937 cells pretreated with IFN-γ*

Final concentration of IgG and $F(ab')_2$ (ug/ml)	Percentage of dengue antigen-positive cells			
	IFN-γ pretreatment		No treatment	
	IgG	$(Fab')_2$	IgG	$(Fab')_2$
None	1.0	1.0	1.4	1.4
0.001	1.3	1.3	1.0	0.5
0.01	6.2	0.9	3.5	1.5
0.1	36.5[a]	2.1	9.8[a]	1.2
1	73.7[a]	0.4	34.2[a]	1.2
10	36.5[a]	<0.3	16.8[a]	<0.3
100	11.6[b]	0.4	7.0[b]	<0.3

*U937 cells were incubated with or without 100 U/ml of rIFN-γ for 24 hours, and infected with dengue virus at m.o.i. of 5 p.f.u./ml in the presence of various concentrations of purified anti-dengue IgG or $F(ab')_2$ prepared from IgG. The percentage of dengue antigen-positive cells was determined 24 hours after infection. The percentage of antigen positive cells was compared between IFN-γ-pretreated cells and nontreated cells at same concentrations of IgG and $F(ab')_2$.

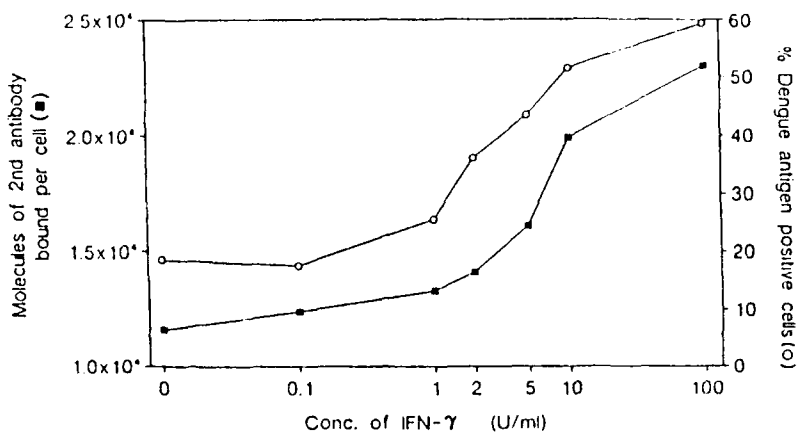
[a] $p<0.001$; [b] $p>0.005$; $p>0.05$ (not significant) at other concentrations of IgG and all the concentrations of $F(ab')_2$.

E-7. Augmentation of dengue virus infection correlates with increase in the number of Fcγ receptors

We tried to determine whether there is a correlation between the number of Fcγ receptors and the percentage of dengue antigen-positive cells. U937 cells were incubated with variable concentrations of IFN-γ for 24 hours, and examined for Fcγ receptor expression by quantitative FACS-analysis after exposure

to MAb 32 which is specific for the human FcγRI. The percentage of antigen positive cells was determined 24 hours after infection. The results shown in Figure 18 demonstrate that there is a good correlation between the percentage of dengue antigen-positive cells and the number of Fcγ receptors. This result is consistent with those shown in Table 34, and indicates that augmentation of dengue virus infection induced by IFN-γ is mediated by Fcγ receptors.

Figure 18. Correlation between IFNγ-increased Fcγ receptors and dengue virus infection.



F. Antibody-dependent enhancement of dengue virus infection mediated by bispecific antibodies

In this study, we examine the mechanisms of ADE by using bispecific antibodies. We prepare bispecific antibodies by chemically cross-linking anti-dengue virus antibodies to antibodies specific for cell surface molecules, including FcγRI, FcγRII, ß2-microglobulin, CD15 and CD33, and examine whether these bispecific antibodies enhance dengue virus infection. Antibody dependent enhancement is mediated by bispecific antibodies targeting dengue virus to FcγRI and FcγRII. Furthermore, bispecific antibodies targeting dengue virus to the other cell surface molecules including ß2-microglobulin, CD15 and CD33, also enhance dengue virus infection.

F-1. Bispecific antibodies targeting dengue virus to FcγRI enhance infection

Previously, FcγRI was shown to mediate ADE in the presence of conventional anti-dengue virus antibodies. Therefore, as an initial test of bispecific antibody-mediated ADE, we investigated whether targeting dengue virus to FcγRI by bispecific antibodies would enhance infection. U937 cells were incubated with IFNγ for 24 hours to up-regulate FcγR expression and infected with dengue virus-bispecific antibody complexes. The percent of dengue virus antigen-positive cells was examined by indirect immunofluorescence

24 hours after infection. The bispecific antibodies 32 x 4G2 and 32 x 2H2 both enhanced dengue virus infection (Table 35, Exp. 1-3). Viral titers from the culture supernatant fluids of U937 cells infected with dengue-bispecific antibody complex (32 x 4G2) were higher than those from the culture fluids of U937 cells infected with dengue virus alone (Table 35, Exp. 2). In the next series of experiments we used A12.13 without IFNγ-pretreatment. A12.13 cells are subclones of U937 cells which have a high expression of FcγRI (20). A12.13 without IFNγ treatment had enhanced infection with bispecific antibody complex 32 x 4G2 (Table 35, Exp. 4).

A bispecific antibody which is specific for Fc RI and Toxoplasma gondii (32 x anti-TG) did not enhance infection (Table 35, Exp. 5). Thus, enhanced infection with 32 x 4G2 and 32 x 2H2 was not due to a nonspecific effect of a bispecific antibody binding to FcγRI. Bispecific antibody 32 x 2H2 did not enhance infection of cells that do not express FcγRI including K562, Molt-3 and Raji cells (data not presented).

Table 35: Enhancement of dengue virus infection by bispecific antibodies targeting dengue virus to FcγRI[a]

	Cells	Antibody	Dilution	% Dengue Antigen Positive Cells[b]	Viral Titer (p.f.u./ml)
Exp. 1	U937	None	--	9.7	
		4G2	$1:4x10^2$	79.4*	
		32x4G2	$1:2x10^2$	47.4*	
Exp. 2	U937	None	--	18.4	$8.0x10^5$
		32x4G2	$1:2x10^2$	52.2*	$2.8x10^6$
Exp. 3	U937	None	--	5.7	
		2H2	$1:4x10^2$	76.6*	
		32x2H2	$1:2x10^3$	19.4*	
Exp. 4	A12.13	None	--	8.2	
		4G2	$1:4x10^2$	81.3*	
		32x4G2	$1:2x10^2$	24.7*	
Exp. 5	A12.13	None	--	6.8	
		2H2	$1:4x10^2$	33.5*	
		32xTg	$1:2x10^1$	4.4NS	
			$1:2x10^2$	6.0NS	
			$1:2x10^3$	5.9NS	

[a]U937 cells were pre-treated with IFNγ (100 u/ml) for 24 hours and then infected with dengue virus or dengue virus antibody complex. A12.13 cells were not pre-treated with IFN before infection.

[b]Chi square analysis was used to compare the cells infected in the presence of anti-dengue virus antibody to those cells infected in the absence of antibody. *$p \leq 0.001$
NS not significant

F-2. Bispecific antibodies targeting dengue virus to FcγRII enhance infection

Conventional antibodies to dengue virus can also mediate ADE through FcγRII. U937 and K562 cells infected with dengue virus complexed to a bispecific antibody directed against FcγRII (IV.3 x 2H2) demonstrated enhancement of dengue virus infection (Table 36, Exp. 1-3). IFNγ which does not modulate the expression of FcγRII on these cells, had no significant effect on the infection of K562 cells with dengue virus-specific antibody complex (IV.3 x 2H2) (Table 36, Exp. 2). Viral titers from the culture supernatant fluids of K562 cells infected with dengue virus-bispecific antibody complex (IV.3 x 2H2) were higher than those from the culture fluids of cells infected with dengue virus alone (Table 36, Exp. 2).

A bispecific antibody linking FcγRII to Toxoplasma gondii (IV.3 x anti-Tg) did not mediate enhancement of dengue virus infection in K562 cells (Table 36, Exp. 3). Furthermore, the bispecific antibody IV.3 x 2H2 did not enhance infection of Molt-3 cells, which do not express Fc RII (data not presented).

Table 36: Enhancement of dengue virus infection by bispecific antibodies targeting dengue virus to FcγRII

	Cells	Antibody	Dilution	% Dengue Antigen Positive Cells[b]	Viral Titer (p.f.u./ml)
Exp. 1	U937[a]	None	--	5.7	
		2H2	$1:4x10^2$	76.6*	
		IV.3x2H2	$1:2x10^1$	9.1*	
			$1:2x10^2$	37.9*	
			$1:2x10^3$	6.2 NS	
Exp. 2	K562	None	--	4.9	$9.0x10^4$
		IV.3x2H2	$1:2x10^2$	15.1*	$3.0x10^5$
	K562[a]	None	--	3.3	$1.6x10^4$
		IV.3x2H2	$1:2x10^2$	18.1*	$3.0x10^5$
Exp. 3	K562	None	--	11.8	
		IV.3x2H2	$1:2x10^2$	50.4*	
		IV.3xTg	$1:2x10^1$	9.0 NS	
			$1:2x10^2$	11.4 NS	
			$1:2x10^3$	11.8 NS	

[a]U937 or K562 cells were pre-treated with IFNγ (100 u/ml) for 24 hours and then infected with dengue virus or dengue virus antibody complex.

[b]Chi square analysis was used to compare the cells infected in the presence of anti-dengue virus antibody to those cells infected in the absence of antibody. *$p \leq 0.001$, NS not significant

F-3. Bispecific antibodies targeting dengue virus to cell surface molecules other than Fcγ receptors enhance infection

The above experiments demonstrated that bispecific antibodies which link dengue virus to FcγRI or FcγRII mimic conventional antibodies in enhancing infection. The advantage of using bispecific antibodies is that they permit targeting to any antigen on the cell surface. Therefore, in order to investigate if the FcγR has a unique function in ADE, infections were performed in the presence of bispecific antibodies targeting dengue virus to non-Fc R molecules.

We examined ADE mediated by a bispecific antibody targeting dengue virus to the β_2-microglobulin of class I major histocompatibility complex (MHC) molecule on U937 cells and the FcγR negative T cell line Molt-3. IFNγ has been shown to up-regulate MHC class I (21) and therefore, both U937 and Molt-3 cells were incubated with IFN before infection. In these cells, targeting dengue virus to β_2-microglobulin with the bispecific antibody BBM1 x 2H2 enhanced infection (Table 37, Exp. 1-5). Viral titers from the culture fluids of Molt-3 cells infected with dengue virus-bispecific antibody complex (BBM1 x 2H2) were higher than those in the culture fluids of cells infected with dengue virus alone (Table 37, Exp. 4). Consistent with the observation that Molt-3 cells do not express Fc receptors, the parent IgG2a mAb, 2H2, did not mediate ADE of these cells (Table 37, Exp. 2).

The control bispecific antibody, BBM1 x anti-Tg, did not mediate enhanced dengue virus infection of Molt-3 cells (Table 37, Exp. 5). Daudi cells, which do not express β_2-microglobulin, did not show enhanced infection in the presence of BBM1 x 2H2 (data not presented). Furthermore, the enhancement of Molt-3 infection mediated by bispecific antibody BBM1 x 2H2 was significantly inhibited by preincubation of these cells with the mAb BBM1.

Table 37: Enhancement of dengue virus infection by bispecific antibodies targeting dengue virus to ß2-microglobulin

	Cells	Antibody	Dilution	% Dengue Antigen Positive Cells[b]	Viral Titer (p.f.u./ml)
Exp. 1	U937[a]	None	--	5.7	
		2H2	$1:4x10^2$	76.6*	
		BBM1x2H2	$1:2x10^1$	13.7*	
			$1:2x10^2$	22.6*	
			$1:2x10^3$	6.3 NS	

Exp. 2	Molt3[a]	None	--	7.4	
		2H2	$1:4x10^2$	2.2*	
		BBM1x2H2	$1:2x10^1$	5.5 NS	
			$1:2x10^2$	20.0*	
			$1:2x10^3$	10.7 NS	
Exp. 3	Molt3	None	--	3.7	
		BBM1x2H2	$1:2x10^2$	9.7**	
	Molt3[a]	None		3.8	
		BBM1x2H2	$1:2x10^2$	20.2*	
Exp. 4	Molt3[a]	None	--	0.8	$2.0x10^2$
		BBM1x2H2	$1:2x10^2$	7.8*	$1.1x10^3$
Exp. 5	Molt3[a]	None	--	5.0	
		BBM1x2H2	$1:2x10^2$	11.2*	
		BBM1xTg	$1:2x10^1$	5.0 NS	
			$1:2x10^2$	4.3 NS	
			$1:2x10^3$	6.2 NS	

[a]U937 or Molt-3 cells were pre-treated with IFNγ(100 u/ml) for 48 hours and then infected with dengue virus or dengue virus antibody complex. The m.o.i. used in Exp. 4 was 14 p.f.u./cell.

[b]Chi square analysis was used to compare the cells infected in the presence of anti-dengue virus antibody to those cells infected in the absence of antibody. *$p \leq 0.001$, **$p \leq 0.05$, NS not significant.

We then examined bispecific antibodies targeting dengue virus to two other molecules, CD15 and CD33, for their ability to enhance infection. HL60 cells infected in the presence of a bispecific antibody which targeted the virus to CD15 (PM81 x 4G2) also displayed enhanced infection demonstrated by an increase in the percentage of dengue Ag-positive cells and by the viral titers (Table 38, Exp. 1 and 2). This enhancement was blocked by preincubation with mAb PM81 but not by mAb Leu 11b.

The bispecific antibody 251 x 4G2 which targets dengue virus to CD33 also showed enhancement of infection of U937 cells demonstrated by the percentage of dengue Ag-positive cells and by the viral titers (Table 38, Exp. 3 and 4). This enhancement was blocked by preincubation of U937 cells with mAb 251, but not mAb 32. Taken together, these results indicate that bispecific antibody-mediated ADE can occur through cell surface molecules other than FcγR.

Table 38: Enhancement of dengue virus infection by bispecific antibodies targeting dengue virus to CD15, and targeting dengue virus to CD33

	Cells	Antibody	Dilution	% Dengue Antigen Positive Cells[a]	Vital Titer (p.f.u./ml)
Exp. 1	HL60	None	--	0.6	
		PM81x4G2	$1:2x10^1$	4.6*	
			$1:2x10^2$	6.6*	
			$1:2x10^3$	14.7*	
Exp. 2	HL60	None	--	0.4	$1.0x10^1$
		PM81x4G2	$1:2x10^4$	13.5*	$2.6x10^3$
Exp. 3	U937	None	--	5.7	
		251x4G2	$1:2x10^1$	4.9 NS	
			$1:2x10^2$	19.2*	
			$1:2x10^3$	16.1*	
Exp. 4	U937	None	--	4.0	$1.1x10^3$
		251x4G2	$1:2x10^2$	22.5*	$1.8x10^4$

[a]Chi square analysis was used to compare the cells infected in the presence of anti-dengue virus antibody to those cells infected in the absence of antibody. *$p \leq 0.001$, NS not significant.

NS not significant

G. Production of interferon by dengue virus-infected cells

G-1. Production of interferon alpha by dengue virus-infected human monocytes

PBMC of non-immune donors were infected with dengue virus, and culture fluids were examined for IFN activity. IFN activity at titers of 40 to 150 U/ml was detected in the culture fluids of dengue virus-infected PBMC, but no IFN activity was detected in samples from uninfected PBMC. Experiments were carried out to determine whether IFN-producing cells were contained in the adherent cell fraction, considered to be monocytes, or in non-adherent fractions. High titers of IFN were detected in the culture fluids of dengue virus-infected monocytes, but not in culture fluids of infected non-adherent cells. Dengue antigen-positive cells were detected only in the dengue virus-infected adherent fractions. Non-adherent cells which were exposed to dengue virus in the same way did not contain dengue antigen-positive cells (data not presented). When monocytes of seven donors were infected and cultured, IFN activity at titers of 100 to 600 U/ml was detected in the culture fluids of dengue-infected monocytes from all donors. No IFN activity was detected in the culture fluid of uninfected monocytes (Table 39).

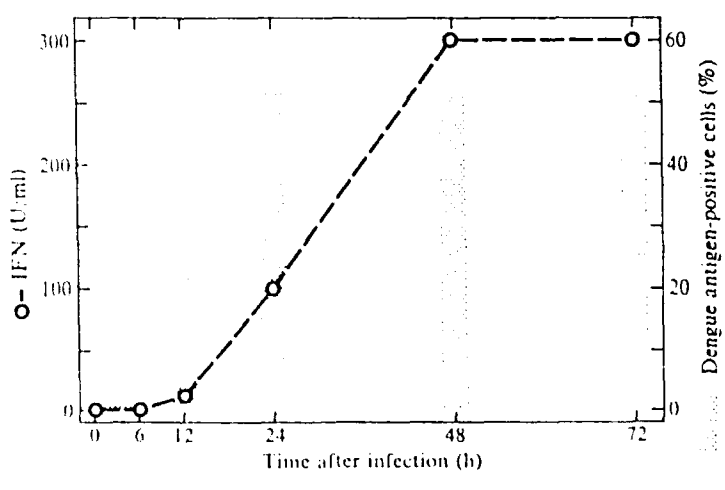
Table 39: Production of IFN by dengue-infected monocytes

Donor	Dengue-infected monocytes* IFN(U/ml)	Dengue-infected monocytes* % Dengue Ag-positive cells	Uninfected monocytes* IFN(U/ml)	Uninfected monocytes* % Dengue Ag-positive cells
A	400	52	<6	0
C	100	22	<6	0
D	400	50	<6	0
E	100	30	<6	0
F	150	42	<6	0
G	600	84	<6	0
H	200	32	<6	0

*Monocytes were infected with dengue virus at m.o.i. of 10 and cultured at a concentration of 1 x 10^6/ml for 48 hours. Culture fluids were collected and examined for IFN activity in bioassays. The percentage of dengue Ag-positive cells was determined by indirect immunofluorescence.

The time course of IFN production and the appearance of dengue antigens in the cytoplasm of infected monocytes was studied. IFN activity was first detected at 12 hours after infection and reached a maximum level by 48 hours. Cytoplasmic dengue antigens were first detected at 6 hours and the percentage of antigen-positive cells reached a maximum level by 24 hours after infection (Figure 19). These results demonstrate a rising titer of IFN production shortly after the appearance of cytoplasmic dengue antigens.

Figure 19. Time course of IFN production and appearance of cytoplasmic dengue antigens in infected monocytes.



The IFN produced was characterized as IFNα, because antiserum to HuIFNα, but not to HuIFNβ or HuIFNγ, neutralized all the IFN activity detected. This result was confirmed by RIA using monoclonal antibodies to HuIFNα and HuIFNγ.

To determine whether the IFNα produced by dengue virus-infected monocytes could inhibit dengue virus infection of other human monocytes, monocytes were incubated for 20 hours in culture fluid obtained from dengue virus-infected monocyte cultures which contained 400 U/ml of IFN , and then they were infected with dengue virus. None of the monocytes treated with culture fluids of dengue-infected monocytes contained dengue viral antigens 24 hours after infection; however, 50% of untreated monocytes and monocytes treated with culture fluids from uninfected monocytes contained dengue viral antigens (Table 40). This result indicates that IFN produced by dengue virus-infected monocytes inhibit infection of monocytes by dengue virus.

Table 40: Inhibition of dengue virus infection of monocytes by induced IFNα

Treatment of monocytes*	IFN titer (U/ml) contained in the culture fluids	% Dengue Ag-positive cells
None (RPMI/10% FCS)	<6	51
Culture fluid of dengue-infected monocytes	400	0
Culture fluid of uninfected monocytes	<6	50

*Supernatant fluids from dengue-infected or uninfected monocyte cultures at a concentration of 1 x 10^6/ml were removed and examined for IFN activity in bioassays. They were then added to other monocyte cultures at a concentration of 1 x 10^6/ml for 20 hrs, prior to infection with dengue virus at an m.o.i. of 10. The percentage of monocytes infected with dengue virus was determined 24 hours after infection by indirect immunofluorescence.

G-2. Production of interferon beta by dengue virus-infected human skin fibroblasts

The supernatant fluids of dengue virus-infected fibroblasts were examined for interferon (IFN) activity. High levels of IFN activity were detected at 24-48 hours after infection (Table 41). IFN was characterized as IFN-β, because specific antibody to IFN-β neutralized the activity, but antibodies to IFN-α or IFN-γ did not.

We examined whether IFN-ß produced by dengue virus-infected fibroblasts protects other fibroblasts from dengue virus infection. Supernatant fluids of dengue virus-infected fibroblasts were exposed to ultra violet light at 4°C for 20 minutes to inactivate dengue virus. Pretreatment of fibroblasts with culture supernatants of dengue virus-infected fibroblasts prevented dengue virus infections (Table 42). Addition of anti-IFN-ß antibody abrogated the ability of culture fluids to protect other fibroblasts from infection. These results confirm that IFN-ß included in the culture fluids is responsible for the prevention of dengue virus infection in other fibroblasts.

Table 41: IFN production by dengue-2 virus-infected fibroblasts*

	Time after infection (hr)	IFN (U/ml)	% dengue Ag-positive cells
Exp. 1	0	<25	0
	4	<25	15
	24	800	93
	44	1200	91
Exp. 2	0	<25	0
	24	200	55
	48	2400	50

*Fibroblasts were infected with dengue-2 virus at an m.o.i. of 10 p.f.u./cell in experiment 1 and 5 p.f.u./cell in experiment 2, and were cultured at 2 x 10^5/ml. IFN activity in the culture fluids and the percentage of dengue antigen-positive cells were assessed at various times after infection.

Table 42: Protection of dengue-2 virus infection of fibroblasts by IFN-ß*

Treatment of fibroblasts	Anti-IFN-ß antibody	% dengue Ag-positive cells
Culture supernatant of dengue-2 virus-infected fibroblasts	-	1
	+	53
IFN-ß (100 U/ml)	-	1
	+	46
None (medium)	-	47
	+	49

*Fibroblasts were incubated with UV-treated supernatant fluids of dengue-2 virus-infected fibroblasts, which contained 100 U/ml of IFN activity, for 24 hours in the presence (+) or absence (-) of anti-IFN-ß antibody. Fibroblasts were then

infected with dengue-2 virus at an m.o.i. of 5 p.f.u./cell and the percentage of dengue antigen-positive cells was assessed using indirect immunofluorescence.

G-3. Production of IL-6 and GM-CSF by dengue-2 virus-infected fibroblasts

We examined whether dengue virus infection induces IL-6, GM-CSF, IL-1α, IL-1ß, TNFα and IFN-γ. High levels of IL-6 and GM-CSF were detected in the supernatant fluids of dengue virus-infected fibroblasts, and IL-6 and GM-CSF were detected at very low levels in the culture fluids of uninfected fibroblasts. Similar levels of IL-1α were detected in the culture fluids of infected and uninfected fibroblasts. IL-1ß, TNFα or IFNγ were not detected in the culture fluids of infected or uninfected fibroblasts. These results indicate that dengue virus-infected fibroblasts produce IL-6 and GM-CSF.

The effects of IL-6 and GM-CSF on dengue virus infection of fibroblasts were examined. Fibroblasts were incubated with recombinant human IL-6 at 1 to 10,000 U/ml, recombinant human GM-CSF at 1 to 2,500 U/ml or mixtures of IL-6 and GM-CSF for 48 hours, and infected with dengue-2 virus. The percentage of dengue virus antigen-positive cells were similar between untreated fibroblasts and those pretreated with IL-6, GM-CSF or mixtures of IL-6 and GM-CSF (data not presented). These results indicate that IL-6 and GM-CSF do not have antiviral or augmenting effects on dengue virus infection of human skin fibroblasts.

H. Induction of interferon alpha from human lymphocytes by autologous, dengue virus-infected monocytes

H-1. IFN induction from PBL by autologous, dengue virus-infected monocytes

To dissect the interaction between PBL and dengue virus-infected monocytes, we cultured PBL and infected monocytes together or separately for 20 hours, and examined the culture fluids for IFN activity. Culture fluids of PBL and those of dengue virus-infected monocytes contained only low titers of IFN activity. When PBL and dengue-infected monocytes were cultured together IFN activities at titers of 200 to 1200 U/ml were detected. Only very low titers of IFN were detected when PBM were cultured with uninfected monocytes (Table 43). Time course study showed that IFN activity was detected as early as 4 hours after the beginning of incubation, and the IFN titers reached a maximum level at 16 hours. To confirm that the IFN detected was produced by PBL, the PBL were treated with a 0.64 ug/ml of actinomycin D and then cultured with dengue virus-infected monocytes. Pretreatment of PBL with actinomycin D decreased the titer of IFN detected from 800 U/ml to 50 U/ml without changing the viability of PBL (Table 44). This result confirmed that the IFN detected in

the cultures containing both PBL and dengue virus-infected monocytes was produced by PBL.

The IFNs produced were characterized by a radioimmunoassay using monoclonal antibodies to IFNα and IFNγ as detector reagents. The predominant IFN induced from PBL by dengue virus-infected monocytes was IFNα; in addition, low titers of IFNγ were also detected in some experiments.

Table 43: Induction of IFN from PBL by dengue virus-infected autologous monocytes (DV-monocytes)*

		IFN (U/ml)					
Culture	Donor	A	B	C	D	E	F
PBL + DV-monocytes**		800	400	200	300	1200	400
PBL + uninfected monocytes		38	50	6	9	<6	<6
PBL		18	50	6	<6	9	<6
DV-monocytes		18	6	18	<6	75	12
Uninfected monocytes		<6	<6	<6	<6	<6	<6

*5 x 10^5 of PBL were cultured with 1 x 10^4 of autologous, dengue virus-infected monocytes for 20 hours. Culture fluids were examined for IFN activity by bioassay.

**The percentages of dengue antigen-positive cells were as follows: donor A, 63%; donor B, 57%; donor C, 40%; donor D, 45%; donor E, 58%; donor F, 49%.

Table 44: Abrogation of IFN production by the treatment of PBM with actinomycin D*

PBL	Inducer Cells	IFN (U/ml)
Untreated	DV-Monocytes**	800
Actinomycin D-treated	DV-Monocytes	50
Untreated	Uninfected Monocytes	6
Actinomycin D-treated	Uninfected Monocytes	12
Untreated	None	12
Actinomycin D-treated	None	6

*5 x 10^5 of PBL were cultured with 1 x 10^4 of inducer cells for 20 hours. PBM were treated with actinomycin D at 0.64 ug/ml as stated in Materials and Methods.

**Percentage of dengue antigen-positive cells was 58%.

H-2. Requirement for cell contact between PBL and dengue virus-infected monocytes for IFN induction

Culture fluids from dengue virus-infected monocytes were examined for the ability to induce IFN from PBL. When PBM were incubated in the culture fluids of dengue virus-infected monocytes for 20 hours, no IFN activity was induced from PBL. These dengue virus-infected monocytes used in the experiment, however, induced 200 U/ml of IFN from PBL when they were cultured with PBL.

Experiments were also performed using a microporous membrane with 0.4 um pores (Costar, Cambridge, MA) to separate the PBL and dengue infected monocytes. When PBL and dengue virus-infected cells were mixed and cultured, high titers of IFN were detected. However, when PBL and dengue virus-infected monocytes were cultured in the same well but separated by the membrane, only a low titer of IFN was detected. These results indicate that cell contact between PBL and dengue virus-infected monocytes were required for IFN induction.

To determine whether IFN induction is restricted by MHC, we analyzed IFN induction using the PBL of two donors who have completely different MHC Class I and Class II antigens. The dengue virus-infected monocytes of each donor induced similar titers of IFN from the PBL of either donor. These results indicate that MHC compatibility is not required for IFN-induction from PBL by dengue virus-infected monocytes.

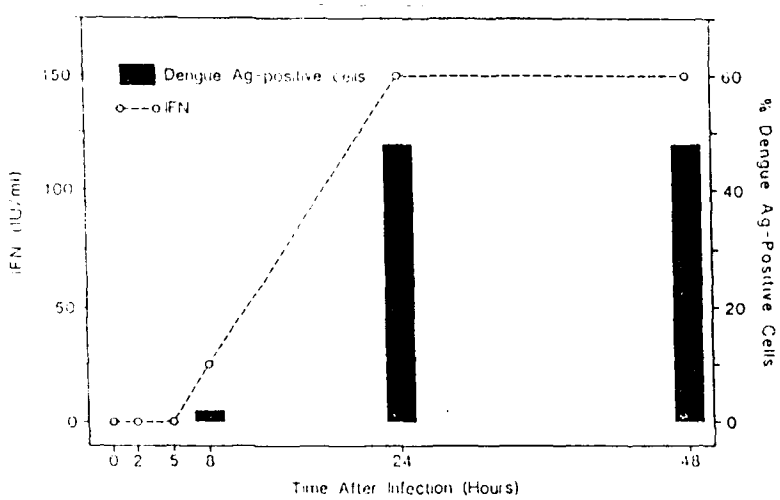
We then examined the ability of paraformaldehyde-treated, dengue virus-infected monocytes to induce IFN. These cells retained the ability to induce IFN from PBL. Glutaraldehyde-treated, dengue virus-infected monocytes also induced IFN from PBL (data not presented). Dengue virus-infected monocytes treated with paraformaldehyde or glutaraldehyde did not produce IFN or infectious dengue virus. These results indicate that production of IFN is due to stimulation of PBL by dengue virus-infected cells and does not directly require infectious dengue virus.

H-3. Correlation between the appearance of dengue antigen in the infected monocytes and IFN-inducing ability

We then analyzed dengue virus-infected monocytes at various times after infection to determine whether there is a correlation between the ability of infected cells to induce IFN and the dengue antigen-expression. At various hours after infection, dengue-infected cells were fixed with paraformaldehyde and used as inducer cells. The infected monocytes were able to induce IFN 8 hours after infection when dengue antigens became detectable. The IFN-inducing ability reached a maximum level 24 hours after infection when the percentage of antigen-positive cells also reached a maximum level (Figure 20). These results indicate that there is a strong correlation between the antigen-expression and

IFN-inducing activity of dengue-infected cells.

Figure 20. Correlation between the appearance of dengue antigen (Ag) in DV-monocytes and IFN-inducing ability.



II-4. Characterization of IFN producing cells using monoclonal antibodies

PBL which produce IFN in response to autologous dengue virus-infected monocytes were characterized using 6 monoclonal antibodies: anti-HLA-DR, OKT3, anti-Leu11, anti-B1, anti-Leu12, and OKM1 antibodies, and one polyclonal anti-human immunoglobulin (IgA + IgG + IgM) antibody. PBL were positively enriched by FACS after reaction with these antibodies and then were cultured with dengue virus-infected monocytes. HLA-DR$^+$ and T3$^-$ cells produced high titers of IFN, while HLA-DR$^-$ and T3$^+$ cells produced very low or undetectable levels of IFN. B cells, characterized as sIg$^+$, B1$^+$, and Leu12$^+$ produced some IFN, while sIg$^-$, B1$^-$, and Leu12$^-$ cells also produced IFN. Natural killer (NK) cells characterized as Leu11$^+$ produced similar titer of IFN as Leu11-cells. M1$^+$ cells, which also include NK cells, produced a higher titer of IFN than M1$^-$ cells (Table 45). These results indicate that IFN producer cells are heterogeneous, but the predominant IFN-producer cells are HLA-DR$^+$ and T3$^-$ cells, and that B and NK cell fractions also include some IFN-producer cells.

Table 45: Characterization of PBL which produce IFN in response to DV-monocytes*

Donor	Lymphocytic fractions	IFN (U/ml) DV-monocytes	Uninfected monocytes	No inducer cells
A	DR+	388	<6	<6
	DR-	38	<6	<6
B	DR+	382	<6	<6
	DR-	7	<6	<6
A	T3+	5	<3	<3
	T3-	100	<3	<3
B	T3+	<6	<6	<6
	T3-	275	<6	<6
B	sIg+	14	<3	<3
	sIg-	200	<3	<3
C	B1+	25	<6	<6
	B1-	175	<6	<6
A	Leu12+	124	<9	<9
	Leu12-	75	<9	<9
B	Leu12+	150	<6	<6
	Leu12-	100	<6	<6
A	Leu11+	100	<6	<6
	Leu11-	100	<6	<6
B	Leu11+	100	6	<6
	Leu11-	150	<6	<6
A	M1+	262	<6	<6
	M1-	37	<6	<6
B	M1+	200	<6	<6
	M1-	75	<6	<6

*PBL were sorted by FACS after reaction with monoclonal antibodies. 3×10^5 of sorted PBL were cultured with 1×10^4 of inducer cells. Purity of FACS-sorted cells was more than 96%. The titers of IFN produced in the dengue virus-infected monocyte cultures without PBL were subtracted from the titers produced in culture which contained PBL exposed to infected monocytes.

H-5. Inhibition of dengue virus-infection of monocytes by induced IFNα

The effect of the induced IFN on dengue virus-infection of monocytes was examined. PBL were cultured with dengue virus-infected monocytes or with uninfected monocytes for 20 hours, and culture fluids were collected and used in the following experiments. Syngeneic monocytes were incubated in the collected culture fluids for 20 hours and the infected with dengue virus. None (0/200) of the monocytes treated with culture fluids from PBL exposed to dengue-infected monocytes, which contained IFN activity at 800 U/ml, contained dengue viral antigens; however, 67% (136/204) of the monocytes treated with culture fluids of PBL which had been exposed to uninfected monocytes contained dengue antigens and a similar percentage (142/212) of monocytes with dengue antigens were observed without addition of supernatant fluids. A reconstitution experiment was performed in which 400 U/ml of Sendai virus-induced IFNα (Interferon Sciences, Inc., New Brunswick, NJ) was added to monocyte culture 20 hrs prior to dengue virus infection. The percentage of dengue virus-infected cells decreased from 48% to 0.8%. These results indicate that IFN produced by PBL after exposure to dengue-infected monocytes inhibits infection of monocytes by dengue virus.

III. DISCUSSION

The role of T lymphocytes in recovery from dengue virus infections and in the pathogenesis of severe complications has not been elucidated. We demonstrated dengue-specific T lymphocyte proliferative responses with PBMC of dengue-antibody positive humans. We used fixed, sonicated dengue virus-infected Vero cells as antigens, as previously were reported with measles virus (22), because cell-free preparations of live dengue virus did not stimulated PBMC of dengue-immune donors (data not presented). Proliferative responses induced by these antigens are dengue-specific, because PBMC of dengue antibody-positive donors respond to dengue antigens but not to control antigen, and PBMC of antibody-negative donors do not respond to dengue or control antigens. Antigens of the four dengue serotypes induced generally similar levels of proliferative responses of PBMC from the Thai adult donors. The dengue infection histories of these adult Thai donors are not known, but they all have high levels of antibodies to the four dengue serotypes, presumably as a result of prior natural infections. The PBMC of an American donor, who had been immunized with yellow fever vaccine and later became infected with dengue 3 virus, responded best to dengue 3 antigen but also responded to dengue 1, 2 and 4 antigens to some degree (data not presented). These results suggest that human T cell responses to primary dengue infections include both type-specific and serotype cross-reactive responses.

The dengue specific proliferating lymphocytes are T cells with CD4 phenotype. These results were obtained using bulk culture stimulated PBMC and dengue-specific T cell lines. It has been reported that CD4+ T cells can be divided into two subsets; helper T cells for antibody production (23) and the inducer of the CD8+ suppressor cells (24). It has also been reported that virus-specific, HLA class II-restricted cytotoxic T cell clones have the CD4 phenotype (25-27). Our results which demonstrate that CD4+ T cells respond to dengue-Vero antigen do not rule out a contribution for CD8+ T cells during dengue virus infections in vivo. It has been reported that the phenotypes and functions of T cells generated in vitro after stimulation with viral antigens may vary, depending on the form of the viral antigens used to stimulate, in the case of human cytomegalovirus (28) and varicella zoster virus (29). Those reports state that cell-free virus preferentially induces the proliferation of CD4+ T cells, and that virus-infected autologous fibroblasts stimulated cytotoxic T lymphocytes (CTL) with a CD8+ phenotype (28,29).

We then reported the establishment of dengue antigen-specific human T cell clones and describe the serotype specificity, IFNγ production and cytotoxic activity of these T cell clones. Dengue antigen-specific T cell clones were established from the PBL of a donor who had been infected with dengue 3 virus. All of the clones have a $CD3^+$, $CD4^+$, $CD8^-$ phenotype. Eight out of twelve clones responded to dengue antigens in a serotype cross-reactive fashion. Four of the clones which we called serotype-specific type responded predominantly to dengue 3 antigen. Proliferative responses in bulk cultures of the PBMC of this donor were primarily dengue 3 serotype-specific, but also contained serotype cross-reactive responses to lower levels. Ther fore, the serotype cross-reactive responses observed in bulk culture experiments appear to reflect the cross-reactive responses detected at the clonal level.

Cytotoxic functions of these T cell clones were examined using dengue 2 virus-infected autologous LCL because epidemiological studies in Thailand have shown that secondary infections with dengue 2 virus induced higher rates of DHF/DSS than did secondary infections with the other serotypes of dengue virus (4). All but one serotype cross-reactive clones lysed dengue 2-infected autologous LCL. These clones did not lyse uninfected LCL or K562 cells. The lysis of dengue-infected cells by the clones examined was inhibited by anti-HLA DP and anti-DQ antibodies; therefore, these serotype cross-reactive cytotoxic T cell clones are HLA class II-restricted. It is known that monocytes are the cells which best support dengue virus infection (3), and monocytic cells with dengue antigens have been observed in DHF/DSS patients (30,31). It has been hypothesized that lysis of dengue-infected monocytes may lead to DHF/DSS (5). Therefore, it is important to learn whether these serotype cross-reactive CTL clones can lyse dengue 2 virus-infected autologous monocytes. Two

of the serotype cross-reactive clones we have examined to date lysed dengue type 2 virus-infected autologous monocytes, but they did not lyse uninfected monocytes (data not presented).

In this paper we have reported that: (i) Dengue virus-specific, human $CD4^+8^-$ T cell clones are heterogeneous with at least six patterns of virus- and serotype-specificities; (ii) HLA-restriction of cytotoxicity by dengue virus-specific $CD4^+$ T cell clones is also heterogeneous with HLA DP, DQ and DR each being used as restriction elements by individual CTL clones. (iii) Seven of the twelve examined clones recognize epitopes on the NS3 protein.

We have reported that most of these T cell clones produce IFNγ after stimulation with dengue Ag, and that IFNγ, which upregulates FcγR, augments dengue virus infection of FcγR-positive monocytic cells in the presence of dengue antibodies. Based on these observations we hypothesize that $CD4^+$ T cells may contribute to the pathogenesis of DHF/DSS by producing IFN and by lysing dengue virus-infected monocytes. Epidemiological studies have shown that DHF/DSS are much more commonly observed during a secondary infection with a different serotype from that of the primary infection (5,32). Although the specificities of the clones may not accurately reflect the specificities of the <u>in vivo</u>, uncloned, dengue virus-specific CTL, the identification of serotype-crossreactive and flavivirus-crossreactive $CD4^+$ T cells supports the possibility that such T cells are activated during secondary infection with a dengue virus of a heterologous serotype and that these T cells may contribute to the pathogenesis of DHF/DSS.

Seven of 13 clones were found to recognize the NS3 protein. This result is consistent with our previous observation that purified NS3 induced a high level of proliferation of PBMC from donor A in bulk cultures. The clones which recognize NS3 are containing truncated dengue virus NS3 genome and with synthetic peptides. It is clear that NS3 is not the only protein which contains $CD4^+$ T cell epitopes, since some of the CTL clones do not recognize either NS3 or NS1. Therefore, identification of other protein(s) which contain epitopes recognized by these T cell clones also remains to be done.

We have observed that the viral proteins recognized by dengue virus-specific murine CTL vary with the mouse strains. Therefore, it is likely that dengue virus-specific T cells from other donors who possess different HLA alleles from those of donor A will recognize different epitopes of dengue virus proteins. To gain a complete understanding of the T cell response to dengue viruses in humans, it will be necessary to define the epitopes recognized by dengue virus-specific T cells from individuals with different MHC antigens as well as from individuals infected with various dengue viruses.

In the second half of our studies on T lymphocytes, we describe serotype cross-reactive $CD8^+$ $CD4^-$ class I-restricted dengue-specific CTL obtained after stimulation with live virus of PBMC from a dengue-immune individual. The evidence that lysis was mediated by class I-restricted $CD8^+$ CTL is the following: 1) antibody and C' treatment of the effectors using anti-CD8 or anti-CD3 but not anti-CD16 or anti-CD4 depleted the killing; 2) antibody to class I but not class II antigens decreased the level of lysis; 3) virus-infected fibroblasts, which do not express MHC class II antigens (33), were specifically lysed. In other viral systems (34,35), the use of live virus or virus-infected fibroblasts has also been necessary to induce class I-restricted $CD8^+$ CTL. This may be due to the requirement for intracellular antigen synthesis sometimes required for optimal presentation of antigen in the context of class I molecules (36,37). Stimulation of PBMC with soluble dengue antigens generated $CD4^+$ class II-restricted CTL. We failed to induce $CD8^+$ CTL with soluble dengue antigens despite inducing good levels of proliferation.

A rather large amount of dengue virus was necessary for optimal CTL induction (10^7-10^8 pfu). During secondary dengue infections, the increased amount of virus present due to antibody-mediated enhancement of infection in Fc-receptor positive cells and the presence of memory CTL may result in a more vigorous CTL response than is usually present during primary infections. This CTL response may contribute to both the enhanced destruction of virus-infected cells and to immunopathology.

In order to postulate that CTL play a role in viral clearance and DHF/DSS during secondary dengue infections, they must be serotype cross-reactive with regard to specificity for induction and lysis. Stimulation by dengue 2 virus of PBMC from a dengue 4-immune individual induced proliferation and CTL capable of lysing dengue 2-infected targets. Stimulation of these PBMC with dengue 4 virus resulted in CTL capable of lysing target cells expressing all 4 serotypes of dengue virus, thus demonstrating the cross-reactive nature of these CTL.

We also described the establishment of dengue virus-specific, serotype cross-reactive, human CD8+ CD4- CTL lines and clones. We utilized these CTL to characterize serotype cross-reactive cytotoxicity, allele specificity, and the epitopes recognized during class I restricted human T cell response to dengue virus infection. All human CTL lines and clones were established from the PBMC of a dengue 4 virus-immune donor obtained two years post-infection. Live dengue 4 virus at high titers in the presence of autologous PBMC was necessary for establishment and maintenance of class I restricted CTL. Several clones and lines were maintained for greater than 1 year in continuous culture using this method. However, the majority of clones grew slowly and were relatively short-lived (<2 months in continuous culture) leaving only a limited number of lines/clones available for advanced

characterization. Class I restricted activity was initially screened for using ^{51}Cr-release cytotoxicity assays with autologous, dengue 2 virus infected fibroblasts as targets. Fibroblasts lack constitutive HLA class II expression in the absence of IFN , and no class II expression was detected by FA analysis using FITC-conjugated anti-class II antibody. The class I restricted nature of 2 lines and 2 clones was further demonstrated by inhibition of lysis of autologous D2V-infected target cells by an anti-class I specific antibody (W6/32), but not by anti-class II specific antibodies directed against DR, DQ, or DP antigens.

Our results indicate that NS3 provides immunodominant epitope(s) for the dengue-virus specific, serotype cross-reactive, class I restricted CTL response in this donor and that multiple epitopes are contained in amino acids 493-618 of NS3. Dengue virus immune responses in more donors need to be characterized to determine the relative contribution of NS3 in generating CTL response to dengue virus infection. The importance of NS3 is supported by other recent work from our laboratory. CTl clones recognizing NS3 epitopes also dominate the dengue virus specific, serotype cross-reactive, class II restricted T cell memory of a dengue 3 virus-immune donor. It is not known why NS3 appears to provide dominant epitopes for memory CTL response to dengue virus infection. Our results suggest that NS3 may contain dominant T cell epitopes for both $CD8^+$ and $CD4^+$ T cell responses. Therefore, NS3 or peptides of NS3 may provide plausible candidates for design of effective dengue virus subunit vaccines.

We found evidence of marked T cell activation in patients with DHF. T cell activation in patients with DF was similar to that seen in other acute febrile diseases, and was not as profound as in patients with DHF.

Among patients with DHF, levels of sIL-2R, sCD4, sCD8, IL-2 and IFNγ were higher than those in the sera of healthy Thai children. it is known from <u>in vitro</u> studies that activated T lymphocytes produce IL-2 (38-40) and IFNγ (38,40), and release sIL-2R (41). Activated $CD8^+$ T lymphocyte release sCD8 (42,43), while activated $CD4^+$ T lymphocytes release sCD4. The high levels of sIL-2R, sCD4, sCD8, IL-2 and IFNγ suggest that $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ T cells observed <u>in vitro</u> are activated <u>in vivo</u> in the acute phase of DHF. Mean levels of sIL-2R, sCD4, sCD8, IL-2 and IFNγ were similarly high among WHO grades 1, 2 and 3 of DHF, and differences in the severity of DHF were not reflected in the apparent degree of T cell activation. However, the absence of difference in the levels of serum factors among WHO grades 1, 2 and 3 of DHF may be partially due to small sample numbers. T cell activation may be different between patients with primary and secondary dengue virus infections manifesting DHF. However, in this study there were only 7 patients with primary DHF, and differences were not detected. Dengue virus serotype might also

influence the degree of T cell activation. However, dengue virus were isolated from only 9 out of 59 patients with DHF; dengue-1 virus from 2 patients, dengue-2 virus from 3 patients and dengue-3 virus from 4 patients. These numbers were too small to determine whether there is any difference in T cell activation among the dengue virus serotypes.

Among patients with DF, the acute sera contained higher levels of sIL-2R, sCD4, IL-2 and IFNγ than the sera of healthy children. SCD8 levels were not elevated in most cases; however, high levels of sCD8 were detected in the small number of hospitalized patients with DF on days 5-8 after onset of fever when sera were available (data not presented). Sera of patients with DHF contained higher levels of sIL-2R, sCD4 and sCD8 than the sera of patients with DF. These results indicate that the levels of activation of $CD4^+$ and $CD8^+$ T lymphocytes are higher in DHF than in DF, and suggest that high levels of T cell activation may be associated with the pathogenesis of DHF. However, the possibility that the higher levels of soluble factors in DHF reflect a high level of inflammatory responsiveness rather than actually causing hemorrhage and plasma leakage cannot be ruled out.

Elevated levels of sIL-2R have been reported in measles (44), HTLV-1 (45) and HIV infections (44-48). Elevated levels of sCD8 have been reported in measles (44) and Epstein-Barr virus infections (43). Elevated levels of IFNγ have been reported in measles (49). We also observed elevated levels of sIL-2R, IL-2 and IFNγ in the sera of Thai children with uncharacterized febrile diseases other than DF, DHF and Japanese encephalitis (data not presented). These results suggest that T lymphocytes are activated during systemic virus infections, and that elevation of serum levels of sIL-2R, sCD4, sCD8, IL-2 and IFNγ is not unique to dengue virus infections.

The role of lymphokines in the pathogenesis of DHF is an interesting subject to be studied. IL-2 induces plasma leakage in humans when administered at doses greater than 10^5 U/kg (50,51). Although the mechanism of the IL-2 induced plasma leakage is not clearly understood, IL-2 is known to induce lymphokine-activated killer (LAK) cells (52) and thromboxane A_2 (53), and activate endothelial cells (54), any of which may conceivably alter endothelial permeability to cause plasma leakage. Activation of the complement system, which is observed in DHF (5), has been observed in patients administered high doses of IL-2 , and the levels of plasma C3a correlated with signs of vascular leak syndrome (55). These observations suggest that the high levels of IL-2 detected in the sera of patients with DHF may be one factor which contribute to plasma leakage and shock in DSS. However, we detected similar levels of IL-2 in the sera of patients with DF, therefore, it is unlikely that IL-2 alone induces DHF.

We have hypothesized that IFNγ produced by dengue specific T cells may contribute to the pathogenesis of DHF (56). It is known that IFN increases the number of Fc RI on monocytic cells (18,29). We have reported that IFNγ augments dengue virus infection of human monocytic cells in the presence of antibody to dengue viruses. IFNγ also upregulates expression of HLA class I and class II antigens (57). These effects of IFNγ may increase the number of dengue virus-infected monocytes and facilitate recognition of dengue virus antigen by dengue virus-specific T cells. The lysis of dengue virus-infected monocytes by these CTL may release vasoactive mediators which contribute to DHF. The elevated levels of IFNγ in the sera of most of the dengue patients suggest that these mechanisms may occur in vivo. However, we detected similar levels of IFNγ in DHF and DF; therefore, IFNγ alone is probably not responsible for the pathogenesis of DHF. It is possible that cytokines released from monocytes may contribute to the pathogenesis of DHF. We did not detect TNFα in the sera of 18 patients with DHF between days 1-11 (data not presented). However, it is interesting to examine serum levels of other monokines in patients with DHF or DF.

We observed significant correlations between the severity of illness (DHF versus DF) and elevated levels of sIL-2R, sCD4 and sCD8, but not with elevated levels of lymphokines. Plasma leakage during dengue virus infections may not be the result of any single lymphokine. Marked activation of T cells does however appear to be a feature of such cases. Therefore, future examination of the interaction of immune effector cells and infected target cells will be important to elucidate the pathogenesis of plasma leakage. Despite our failure to detect a correlation between serum levels of lymphokines and plasma leakage, we want to emphasize that the patients we investigated were admitted to the hospital when symptoms were already severe or rapidly worsening. It is possible that many of the host immune responses had been already activated and some of the secreted lymphokines had been eliminated or degraded before sera were obtained. It would be desirable to serially evaluate a cohort of patients beginning earlier in the course of dengue virus infection to determine whether elevated serum levels of specific lymphokines predict the appearance of plasma leakage.

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